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(54) Title: PROCESS FOR MODIFYING PLANTS

(57) Abstract: The use of a gene expressing a non-feed back inhibited HMG-reductase to increase the level of 4-desmethylsterols in the seeds of plants.

PROCESS FOR MODIFYING PLANTS

Field of the invention

The invention relates to a process for the modification of plants, more specifically a process for increasing the isoprenoid level in plants.

Background of the invention

Many approaches have been suggested for modifying the isoprenoid production in plants.

Whereas only a few sterols exist in animals, with cholesterol being by far the major one, in plants a wide range of sterols are found. Structural variations between these arise from different substitutions in the side chain and the number and position of double bonds in the tetracyclic skeleton. Plant sterols can be grouped by the presence or absence of one or more functionalities. For example they can be divided into three groups based on methylation levels at C4 as follows: 4-desmethylsterols or end product sterols, 4α-monomethylsterols and 4,4-dimethylsterols. Naturally occurring 4-desmethylsterols include sitosterol, stigmasterol, brassicasterol, Δ7-avenasterol and campesterol. In most higher plants, sterols with a free 3β-hydroxyl group (free sterols) are the major end products. However sterols also occur as conjugates, for example, where the 3-hydroxy group is esterified by a fatty acid chain, phenolic acids or sugar moieties to give steryl esters. For the purpose of this description the term sterol refers both to free sterols and conjugated sterols. However

in this specification references to levels, amounts or percentages of sterol refer to the total weight sterol groups whereby the weight of the conjugating groups such as fatty acid, phenolic acid or sugar groups is excluded.

To date most studies aimed at manipulating sterols in plants have involved other than 4-desmethylsterols with the purpose of increasing resistance to pests or to fungicides.

WO 98/45457 describes the modulation of phytosterol compositions to confer resistance to insects, nematodes, fungi and/or environmental stresses, and/or to improve the nutritional value of plants by using a double stranded DNA molecule comprising a promoter, a DNA sequence encoding a first enzyme which binds a first sterol and produces a second sterol and a 3' non-translated region which causes polyadenylation at the 3' end of the RNA. Preferably the enzyme is selected from the group consisting of S-adenosyl-L-methionine-$\Delta^{24(25)}$-sterol methyl transferase, a C-4 demethylase, a cycloeucalenol to obtusifoliol-isomerase, a 14-α-demethylase, a Δ^{8} to Δ^{7}- isomerase, a Δ^{7}-C-5-desaturase and a 24,25-reductase.

US 5,306,862 describes a method of increasing sterol accumulation in a plant by increasing the copy number of a gene encoding a polypeptide having HMG-CoA reductase activity to increase the resistance of plants to pests. Similarly US 5,349,126 discloses a process to increase the squalene and sterol accumulation in transgenic plants by increasing the amount of a gene encoding a polypeptide having HMG-CoA reductase activity to increase the pest resistance of transgenic plants.

WO 97/48793 discloses a C-14 sterol reductase polypeptide for the genetic manipulation of a plant sterol biosynthetic pathway.

WO 96/09393 discloses a DNA sequence encoding squalene synthetase.

WO 97/34003 discloses a process of raising squalene levels in plants by introduction into a genome of a plant a DNA to suppress expression of squalene epoxidase.

WO 93/16187 discloses new plants containing in its genome one or more genes involved in the early stages of phytosterol biosynthesis, preferably the genes encode mevanolate kinase.

US 5,589,619 discloses accumulation of squalene in plants by introducing a HMG-CoA reductase gene to increase production of sterol and resistance to pests. Example 10 discloses increased squalene levels in the seeds of these plants.

In plants, mevalonate synthesis via HMGR is one of the steps in isoprenoid biosynthesis.

Gondet et al in Plant Physiology (1994) 105:509-518 has isolated a tobacco mutant showing dramatically altered sterol compositions in leaf tissue with significant increases in the proportion of cyclopropylsterols and HMGR activities increased by approximately 3-fold.

Re et al in The Plant Journal (1995) 7(5), 771-784 have shown that the over-expression of HMG CoA reductase is not sufficient to alter the bulk synthesis and accumulation of end product of the plant isoprenoid pathway.

Applicants believe that the reason for this is that the activity of HMGR in plants is subject to feedback inhibition by sterols. Some HMGR genes, however are non-feed back inhibited. Examples of such genes are non-plant HMGR genes lacking the membrane binding domain such as the truncated hamster HMGR genes or the truncated *Saccharomyces cerevisiae* genes, and HMGR genes (or truncated versions thereof) from high isoprenoid producing plants such as *Hevea brasiliensis*.

A truncated hamster HMGR gene, lacking the membrane binding domain, was expressed in tobacco plants under the control of the CaMV 35S promoter (Chappell *et al.*, Plant Physiology (1995) 109: 1337-1343). This resulted in a 3- to 6- fold increase in total HMGR activity in leaf tissue.

Schaller et al in Plant Physiology (1995) 109:761-770 discloses the introduction of a HMGR1 gene from *Hevea brasiliensis* into tobacco leading to an enhanced sterol production especially of cycloartenol in leaf tissue.

Polakowski et al in Applied Microbial Biotechnology (1998) 59:66-71 describes the use of a truncated *Saccharomyces cerevisiae hmg 1* gene in yeast, leading to the accumulation of squalene.

The present invention aims to increase sterol levels in plants, whereby the sterols are preferably nutritionally attractive 4-desmethylsterols such as sitosterols,

stigmasterols, brassicasterol, Δ7-avenasterol or campesterols and whereby the sterols are preferably expressed in the seeds.

It has been found that genes expressing specific HMG-reductase enzymes can advantageously be used to increase the nutritional value of plants especially in the seeds thereof. Surprisingly it has been found that the use of non feedback regulated HMGR leads to the enhancement of nutritionally beneficial sterol for example in the seeds of said plants. Surprisingly it has also been found that particularly high levels of sterols can be obtained by using truncated plant HMGR genes.

Statement of the invention

Accordingly the invention relates to the use of a gene expressing a non-feed back inhibited HMG-reductase to increase the level of 4-desmethylsterols in the seeds of plants. Preferably the gene expressing a non-feed back inhibited HMG-reductase is a truncated plant HMGR gene.

Accordingly in a second aspect the invention relates to a method to produce plants having a modified sterol production by incorporating into the plant genome a heterologous gene whereby said gene expresses a truncated plant HMG-reductase.

In a third aspect the present invention relates to modified plants having incorporated in their genome a heterologous gene expressing a truncated plant HMG-reductase.

Detailed description of the invention

In higher plants, isoprenoids are a large family of compounds with diverse roles. They include sterols, the plant hormones gibberellins and abscisic acid, components of photosynthetic pigments, phytoalexins and a variety of other specialised terpenoids.

Sterols, especially 4-desmethylsterols are of interest and colour of fruits and vegetable oils. Of particular interest are isoprenoid compounds of nutritional benefit such as fat soluble sterols. These may be efficacious in reducing coronary heart disease, for example, some phytosterols have been shown to lower serum cholesterol levels when increased in the diet.

Expression of such compounds in plant seeds in particular in oilseeds is commercially advantageous as generally the harvesting of such ingredients from seeds is very convenient and in some instances it may be possible to extract the oil in combination with the sterols from the seed, leading to an oil containing elevated levels of sterol without or with the reduced need for separate addition of sterols.

Preferred sterols are 4-desmethylsterols, most preferred sitosterol, stigmasterol, brassicasterol, avenasterol and campesterol. Also preferably at least part of the sterols, for example at least 50 wt% based on the total of the sterols in the seed are esters of sterols with C10-24 fatty acids. In a very preferred embodiment the sterols comprise C10-24 esters of 4-desmethylsterols.

As discussed above, several approaches have been suggested to alter the levels of isoprenoids in plants. It has now been found that for the enhancement of isoprenoid levels in seeds a preferred route is to use a non feedback inhibited HMGR gene. The use of such genes is especially advantageous to enhance the levels of 4-desmethylsterols, even more preferred the level of stigmasterol, sitosterol and campesterol in plant tissue for example seeds. Also the use of such genes is especially advantageous to enhance the levels of isoprenoids in plant tissue such as oilseeds containing more than 10 wt% based on dry weight of triglycerides.

In a first embodiment of the invention the non-feed back inhibited HMG reductase is an enzyme which is expressed by a truncated non-plant HMGR gene, said truncation preferably leading to an enzyme lacking the membrane binding domain, but whereby the HMGR functionality of the gene is preferably maintained. Examples of such genes are the truncated hamster or yeast HMGR genes.

A second -preferred- embodiment of a non-feedback inhibited HMG reductase is an enzyme expressed by HMGR genes from high isoprenoid producing plants such as *Hevea brasiliensis*. Especially preferred are truncated versions of HMGR produced by genes from high isoprenoid producing plants such as *Hevea brasiliensis*, most preferred truncated versions are used whereby said HMGR lacks the membrane binding domain.

The intact HMGR enzyme comprises three regions: a catalytic region, containing the active site of the enzyme, a

membrane binding region, anchoring the enzyme to the endoplasmic reticulum and a linker region joining the catalytic and membrane binding regions of the enzyme. The membrane-binding domain occupies the N-terminal region of the enzyme, whereas the catalytic region occupies the C-terminal region. It is believed that feedback inhibition in most plants generally requires the presence of the membrane-binding region of the enzyme. Therefore a preferred embodiment of the invention relates to the use of a HMGR gene expressing an enzyme with an inactivated or without a membrane binding domain, whereby said gene is preferably used to increase the level of 4-desmethylsterols in plant tissue such as the seeds of plants.

An example of HMG reductase with an inactivated or without a membrane binding domain is the HMG reductase expressed by the truncated hamster HMGR gene as described by Chappell (see above). The truncation is believed to remove the membrane binding domain from the HMG reductase whereafter a significant reduction of feedback inhibition occurs. Other truncated or mutated genes whereby the membrane binding domain is removed or inactivated can equally be used. An example of this is the truncated HMGR gene as used by Polakowski (see above).

Preferred examples of HMG reductases are those expressed by HMGR genes obtained from plants which naturally have the tendency to develop high levels of isoprenoids such as for example triterpenes and rubber. Examples of such plants are *Asteraceae*, especially *Euphorbiaceae*. Therefore another preferred embodiment of the invention relates to the use of a HMGR gene isolated from *Asteraceae* to increase the level

of sterols, particularly 4-desmethylsterols in plant tissue, particularly the seeds of plants. Preferably the HMGR gene is isolated from *Hevea brasiliensis*. Especially preferably truncated versions of such plant genes may be used.

The invention also provides a method of transforming a plant by

a) transforming a plant cell with a recombinant DNA construct comprising a DNA segment encoding a polypeptide with non feedback inhibited HMGR activity and a promoter for driving the expression of said polypeptide in said plant cell to form a transformed plant cell.

b) regenerating the transformed plant cell into the transgenic plant.

Preferably this method is using a construct comprising a DNA segment derived from plants, particularly a DNA segment encoding a HMG-reductase derived from *Asteraceae*, most preferred a truncated plant HMG-reductase for example a truncated HMG-reductase derived from *Asteraceae* especially *Hevea brasiliensis*.

Furthermore this method preferably involves selecting transgenic plants that have enhanced levels of sterols particularly 4-desmethylsterols in plant tissue particularly in the seeds compared to wild type strains of the same plant.

DNA segments encoding non feedback inhibited HMGR for use according to the present invention may suitably be obtained from animals, microbial sources or plants, Alternatively,

equivalent genes could be isolated from gene libraries, for example by hybridisation techniques with DNA probes.

The gene sequences of interest will be operably linked (that is, positioned to ensure the functioning of) to one or more suitable promoters which allow the DNA to be transcribed. Suitable promoters, which may be homologous or heterologous to the gene, useful for expression in plants are well known in art, as described, for example, in Weising et al, (1988), Ann. Rev. Genetics, 22, 421-477). Promoters for use according to the invention may be inducible, constitutive or tissue-specific or have various combinations of such characteristics. Useful promoters include, but are not limited to constitutive promoters such as carnation etched ring virus (CERV), cauliflower mosaic virus (CaMV) 35S promoter, or more particularly the double enhanced cauliflower mosaic virus promoter, comprising two CaMV 35S promoters in tandem (referred to as a "Double 35S" promoter).

It may be desirable to use a tissue-specific or developmentally regulated promoter instead of a constitutive promoter in certain circumstances. A tissue-specific promoter allows for overexpression in certain tissues without affecting expression in other tissues. By way of illustration, a preferred promoter used in overexpression of enzymes in seed tissue is an ACP promoter as described in WO92/18634.

The promoter and termination regulatory regions will be functional in the host plant cell and may be heterologous (that is, not naturally occurring) or homologous (derived

from the plant host species) to the plant cell and the gene. Suitable promoters which may be used are described above.

The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions which may be used are well known in the art and include *Agrobacterium tumefaciens* nopaline synthase terminator (Tnos), *Agrobacterium tumefaciens* mannopine synthase terminator (Tmas) and the CaMV 35S terminator (T35S). Particularly preferred termination regions for use according to the invention include the pea ribulose bisphosphate carboxylase small subunit termination region (TrbcS) or the *Tnos* termination region.

Such gene constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium* and screening for increased isoprenoid levels.

Suitably, the nucleotide sequences for the genes may be extracted from the Genbank nucleotide database and searched for restriction enzymes that do not cut. These restriction sites may be added to the genes by conventional methods such as incorporating these sites in PCR primers or by sub-cloning.

Preferably the DNA construct according to the invention is comprised within a vector, most suitably an expression vector adapted for expression in an appropriate host (plant) cell. It will be appreciated that any vector which is capable of producing a plant comprising the introduced DNA sequence will be sufficient.

Suitable vectors are well known to those skilled in the art and are described in general technical references such as Pouwels et al, Cloning Vectors. A laboratory manual, Elsevier, Amsterdam (1986). Particularly suitable vectors include the Ti plasmid vectors.

Transformation techniques for introducing the DNA constructs according to the invention into host cells are well known in the art and include such methods as micro-injection, using polyethylene glycol, electroporation, or high velocity ballistic penetration. A preferred method for use according to the present invention relies on agrobacterium - mediated transformation.

After transformation of the plant cells or plant, those plant cells or plants into which the desired DNA has been incorporated may be selected by such methods as antibiotic resistance, herbicide resistance, tolerance to amino-acid analogues or using phenotypic markers.

Various assays may be used to determine whether the plant cell shows an increase in gene expression, for example, Northern blotting or quantitative reverse transcriptase PCR (RT-PCR). Whole transgenic plants may be regenerated from the transformed cell by conventional methods. Such transgenic plants having improved isoprenoid levels may be propagated and self-pollinated to produce homozygous lines. Such plants produce seeds containing the genes for the introduced trait and can be grown to produce plants that will produce the selected phenotype.

Preferably the level of sterols, especially the level of 4-desmethyl sterols in the plant and preferably in the seeds of the plants is at least 5wt% more than the level in corresponding plants without the non-feedback inhibited HMGR gene, more preferred more than 10% more, especially preferred more than 15 % more, most preferred more than 25% more. In a very advantageous embodiment the level of desmethyl sterols is at least 2 times the level in unmodified plants, more preferred at least 5 times. Especially preferably the level of sterols in plant tissue e.g. in leaves or seeds is more than 0.500 wt% based on dry weight.

Another advantage of the current invention is the enhancement of the level of esterified sterols. Most preferably at least 50% of the sterols are in esterified form, more preferred more than 60%.

Suitable plants to be modified may be selected from a wide range. Preferably edible plants are modified, for example plants having edible parts (e.g. vegetables such as cabbage, spinach, lettuce, broccoli, tomato, corn and wheat) or plants having edible fruits (e.g. palm oil trees, tomato plants, fruit trees etc) and plants having edible or extractable seeds (e.g. nut trees, oilseed plants such as soy, rapeseed and sunflower). Preferably the modified plants are oilseed plants such as sunflower, rapeseed and soy or plants having oily fruits such as palm trees or leaf vegetables such as lettuce and spinach.

The invention also provides seeds obtained from oil plants with a non-feedback-inhibited HMGR gene, especially preferred oilseeds are tobacco seeds, canola seeds,

rapeseed, sunflower seed. Also provided is a method to extract oil, whereby the oil is extracted from these seeds. Any suitable method can be used for such extraction.

The invention also provides plant tissue from plants with a non-feedback inhibited heterlogous plant HMGR gene expressing a truncated HMG-reductase. Suitable plant tissue may be leaves, stems, fruits, seeds, flowers or combinations thereof.

The invention will now be further illustrated in the following examples.

Example 1 Transformation of tobacco with *Hevea brasiliensis hmg 1* cDNA

A binary plasmid pHEV 36 containing a 2.1 kb cDNA of *Hevea brasiliensis hmg 1* (accession number X54659) in pMON 9818 (Cuozzo et al, Biotechnology (1988) 6: 549) was obtained from Nam Chua, Rockefeller University, New York (Figure 1).

Binary vector was transformed into *Agrobacterium tumefaciens* pGV3850 using triparental mating as described in Rogers et al 1988: Use of co-integrating Ti-plasmid vectors in Plant Molecular Biology Manual, eds Galvin & Schilperoort, Kluwer Academic Press. Transformants were analysed for presence of the gene of interest by PCR.

PCR positive cultures were used to inoculate a 10 ml Lennox media broth containing kanamycin 50 µg/ml and rifampicin 50 µg/ml. The overnight culture was spun down at 3000g and resuspended in an equal volume of MS media (3% sucrose). Leaf segments were cut from young *Nicotiana tabacum L. cv. SR1* leaves from plants grown in tissue culture. Segments were placed directly into the agrobacterium solution and left for 10 minutes. The segments were then removed and placed upper surface down on feeder plates (10 per plate) and left for 2 days in low light at 22°C. The leaf segments were then placed on tobacco shooting media with hormones containing cefotaxime 500 µg/ml and kanamycin 50 µg/ml with the upper surface up and placed in a growth room at 24°C with a 16hrs light 8 hrs dark regime. Three weeks later the callusing segments were transferred to tubs of tobacco shooting media. Once formed shoots were excised and placed on tobacco shooting media without hormones containing

cefotaxime 500 µg/ml and kanamycin 50 µg/ml to root. Rooted plants were then potted up into a 50% perlite 50% compost mixture and placed in a propagator. After 1 week the plants were removed from the propagator and subsequently potted up into 5 inch pots. Once flowering had begun paper bags were placed over the flowers to prevent cross pollination. When flowering had finished and pods formed, the bags were removed and the amount of water supplied reduced. Seed was harvested from dry pods and stored for subsequent analysis.

Example 2 Sterol Analysis of transgenic tobacco seeds

The plant tissue obtained in accordance to example 1 is freeze dried, then ground to a fine powder. 250µl of 0.2 % w/v dihydrocholesterol dissolved in chloroform is pipetted into a screw-top septum vial. After removal of solvent, an amount of the plant tissue (50 mg) is added to the vial, and total lipid extracted with 5 ml of a 2:1 v/v mixture of chloroform:methanol. The vial is capped and placed in a hot block maintained at 80-85°C. After 30 minutes the contents are filtered and the vial is washed out with a second 5ml aliquot of the chloroform:methanol mixture. The contents of the vial are filtered once more and the filtrates combined. The solvent portion of the filtrate is blown off using a stream of nitrogen gas to isolate the lipid residue.

The lipid fraction is then subjected to transmethylation by heating at 80-85°C in 1 ml of toluene and 2 ml of 0.5N sodium methoxide in methanol. After 30 minutes, 2 ml of a 14 % boron trifluoride solution in methanol is added and heated for a further 10 minutes at 80-85°C. After cooling, 2-3 ml of diethyl ether followed by 5 ml of deionised water

are added. The ether fraction is removed and a further ether extraction carried out. The ether fractions are combined, backwashed with approx. 5 ml of water and dried overnight over anhydrous sodium sulphate. The ether phase is filtered and the solvent removed using a stream of nitrogen gas.

Sterols are dissolved in 300-400 µL of toluene and silylated by the addition of 200 µl of 95:5 N,O-bis(trimethylsilyl)acetamide:trimethylchlorosilane followed by incubation at 50°C for 10 minutes. GC analysis is carried out using a 25 m x 0.32 mm i.d. (0.25 µm film thickness) 5% BPX5 column (ex SGE) in a Perkin-Elmer 8420 GC. The temperature program is 180-240°C at 10°C/min, followed by 240-355°C at 15°C/min. and, finally, 5 min. at 355°C. The FID temperature is 380°C and the helium pressure 10 psi. A volume of 1.0 µl is injected onto the column. A GC response factor of 1.0 for each of the sterols with respect to the dihydrocholesterol internal calibrant is assumed.

The five main sterol peaks (cholesterol, campesterol, stigmasterol, β-sitosterol, isofucosterol) and the intermediate compound cycloartenol were identified by comparison with authentic samples and library spectra following GC-MS analysis (Hewlett Packard 5890 Series 2 Plus GC interfaced to a 5972A mass selective detector) using a 30m x 0.25mm i.d. (0.25 µm film thickness) HP5-MS column. The oven temperature program was 100-320°C at 10°C/min, then 8 min. at 320°C. Electron impact spectra were recorded at 70 eV and an electron multiplier voltage of 2494 V. A helium flow rate of 1ml/min at constant flow

and a 1.0 µl splitless injection were employed. The MS data range was 65-520 Daltons.

The reproducibility of this methodology was confirmed by repeated analysis of a particular batch of wild type tobacco seed. The amount of each sterol in plant tissue is expressed as a percentage of the dry sample weight.

Table 1 shows the sterol analysis of mature seeds obtained from tobacco transformed with *H. brasiliensis hmg1* cDNA. Seeds from 38 independent transgenic plants (HMGR) were analysed along with seeds from 8 independent untransformed plants (SR1) which had been generated via tissue culture. The total sterol content of the SR1 control seeds ranged from 0.364%-0.386% dry weight with a mean of 0.374 (S.D. 0.0072). The HMGR transgenic seeds contained total sterol contents of up to 0.439% which corresponds to increases of up to 17.4% compared to the mean of control seeds. 25 of the 38 HMGR transgenic plants contained total sterol contents above the control mean.

Table 1: Hevea (rubber) HMGR cDNA in tobacco - mature seed analysis

Total sterols as % of dry weight

Sample	Cholesterol	Campesterol	stigmasterol	Sitosterol	Isofucosterol	cycloartenol	Total sterols
HMGR2 49	0.0334	0.0585	0.0420	0.1684	0.0832	0.0539	0.439
HMGR2 16	0.0376	0.0580	0.0365	0.1592	0.0844	0.0551	0.431
HMGR2 43	0.0293	0.0607	0.0395	0.1660	0.0796	0.0540	0.429
HMGR2 36	0.0268	0.0584	0.0419	0.1913	0.0749	0.0291	0.422
HMGR2 11	0.0296	0.0568	0.0382	0.1627	0.0806	0.0540	0.422
HMGR2 48	0.0283	0.0580	0.0403	0.1628	0.0784	0.0474	0.415
HMGR2 14	0.0279	0.0596	0.0401	0.1639	0.0752	0.0455	0.412
HMGR2 25	0.0287	0.0552	0.0368	0.1637	0.0802	0.0469	0.411
HMGR2 23	0.0289	0.0545	0.0367	0.1599	0.0754	0.0535	0.409
HMGR2 27	0.0267	0.0559	0.0388	0.1618	0.0754	0.0494	0.408
HMGR2 10	0.0272	0.0546	0.0398	0.1579	0.0761	0.0522	0.408
HMGR2 12	0.0255	0.0545	0.0370	0.1625	0.0728	0.0512	0.404
HMGR2 32	0.0309	0.0538	0.0354	0.1532	0.0804	0.0492	0.403
HMGR2 2	0.0363	0.0529	0.0347	0.1562	0.0848	0.0355	0.401
HMGR2 52	0.0295	0.0555	0.0383	0.1593	0.0767	0.0372	0.397
HMGR2 3	0.0266	0.0532	0.0385	0.1562	0.0732	0.0378	0.386
HMGR2 37	0.0253	0.0543	0.0371	0.1544	0.0702	0.0443	0.386
HMGR2 9	0.0264	0.0529	0.0383	0.1557	0.0686	0.0435	0.385
HMGR2 35	0.0262	0.0516	0.0372	0.1565	0.0718	0.0408	0.384
HMGR2 8	0.0253	0.0556	0.0358	0.1549	0.0738	0.0383	0.384
HMGR2 6	0.0291	0.0518	0.0354	0.1576	0.0785	0.0288	0.381
HMGR2 50	0.0278	0.0519	0.0332	0.1531	0.0783	0.0362	0.381
HMGR2 7	0.0288	0.0492	0.0349	0.1532	0.0756	0.0358	0.377
HMGR2 42	0.0266	0.0528	0.0373	0.1607	0.0734	0.0264	0.377
HMGR2 53	0.0299	0.0528	0.0345	0.1528	0.0756	0.0298	0.375
HMGR2 1	0.0285	0.0519	0.0376	0.1490	0.0726	0.0336	0.373
HMGR2 55	0.0289	0.0515	0.0371	0.1532	0.0681	0.0314	0.370
HMGR2 5	0.0320	0.0488	0.0349	0.1452	0.0774	0.0302	0.368
HMGR2 45	0.0274	0.0535	0.0377	0.1500	0.0678	0.0313	0.368
HMGR2 54	0.0291	0.0505	0.0346	0.1493	0.0746	0.0286	0.367
HMGR2 29	0.0220	0.0503	0.0385	0.1494	0.0613	0.0422	0.364
HMGR2 31	0.0261	0.0509	0.0325	0.1530	0.0700	0.0304	0.363
HMGR2 26	0.0309	0.0486	0.0326	0.1475	0.0708	0.0313	0.362
HMGR2 46	0.0293	0.0388	0.0321	0.1533	0.0748	0.0305	0.359
HMGR2 56	0.0314	0.0514	0.0381	0.1421	0.0724	0.0224	0.358
HMGR2 44	0.0292	0.0519	0.0320	0.1407	0.0726	0.0276	0.354
HMGR2 38	0.0197	0.0490	0.0397	0.1456	0.0510	0.0375	0.342
HMGR2 30	0.0195	0.0475	0.0371	0.1384	0.0552	0.0375	0.335
SR1 4(control)	0.0276	0.0503	0.0364	0.1528	0.0721	0.0396	0.379
SR1 5(control)	0.0297	0.0517	0.0368	0.1526	0.0784	0.0336	0.383
SR1 6(control)	0.0290	0.0499	0.0346	0.1439	0.0754	0.0317	0.364
SR1 7(control)	0.0272	0.0550	0.0390	0.1481	0.0726	0.0260	0.368
SR1 8(control)	0.0324	0.0547	0.0405	0.1468	0.0744	0.0369	0.386
SR1 10(control)	0.0256	0.0503	0.0419	0.1483	0.0731	0.0354	0.375
SR1 12(control)	0.0251	0.0508	0.0383	0.1531	0.0712	0.0333	0.372
SR1 13(control)	0.0322	0.0501	0.0354	0.1445	0.0762	0.0304	0.369

Example 3 Assay of HMGR activity in transgenic tobacco seeds

Tobacco seeds were collected 18-19 days after anthesis and extracts were prepared by homogenising seeds in 200mM potassium phosphate pH 7.5, 0.35M sorbitol, 10mM EDTA, 5mM $MgCl_2$, 5mM glutathione and 4g/l PVPP in a ratio of 1:2 (seeds:buffer w/v). Total homogenate was assayed immediately for HMGR activity according to the method of Chappell et al Plant Physiol (1995) 109: 1337, except TLC analysis was performed as described by Schaller et al (1995) Plant Physiol 109: 762.

Seeds from two plants with enhanced levels of sterol (HMGR2 and HMGR36 of table 1) were assayed for HMGR activity along with seeds from two control plants (SR4 and SR5 of table 1). Table 2 shows that the two transgenic seed extracts contain significantly higher activities of HMGR compared to control plants. Thus expression of a 'deregulated' form of an HMGR gene enhances the overall HMGR activity in seed tissue leading to elevated levels of seed sterols.

Sample	HMGR activity (pmol/hr/mg seed)
HMGR2 36	2,520
HMGR2 2	2,480
SR1 4	1,780
SR1 5	1,220

Table 2: HMGR activity of trangenic seeds compared to control

Example 4 Transformation of tobacco with another *Hevea brasiliensis hmgr 1* cDNA construct

Hevea brasiliensis hmg 1 cDNA was placed under control of the double Cauliflower Mosaic Virus 35S (2x35S) promoter and, to terminate transcription, the pea ribulose bisphosphate small subunit terminator (TRBCS) has been placed down stream of the *hmg* 1 gene. The chimaeric gene was cloned into a pGPTV- KAN [Becker et al Plant Mol Biol (1992) 20: 1195-97] based binary vector, SJ 34.

Plasmids CJ151, CJ157, PP5LN and SJ34 are shown in Figures 2 to 5. *E. coli* strain DH5α (Gibco BRL) was used as the host strain in all cloning procedures. Bacteria were cultivated in LB medium (10 g/l tryptone, 5g/l yeast-extract, 5 g/l NaCl) supplemented with the appropriate selection pressure (ampicillin (100 µg/ml) or kanamycin (50 µg/ml) on a rotary shaker (210 rpm) at 37 °C.

Plasmid CJ157 was digested with *Hind*III and *Nco*I to obtain the CERV promoter fragment. This fragment was inserted in the corresponding sites of plasmid PP51N resulting in plasmid pNH1. A *Sal*I containing DNA linker was assembled by mixing 4 µmol of oligonucleotides Sal1 and Sal2 with annealing buffer (10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol, Tris-HCl pH 7.5) in 100 µl water. The mixture was heated to 80 °C in a 5 L water bath and cooled down to room temperature over night. The synthetic linker holding the *Sal*I site was inserted between the *Eco*RI and *Xba*I sites of pNH1 yielding pNH2. Oligonucleotides Xma1 and Xma2 were also assembled using the above outlined protocol

rendering a DNA-linker containing a *XmaI* site. The synthetic linker holding the *XmaI* site was inserted between the *HindIII* and *ClaI* site of pNH2 rendering pNH3. Plasmid CJ151 was digested with *ClaI* and *NcoI* to obtain a 785 base pair fragment containing the 2x35S promoter. This fragment was inserted into the corresponding sites of pNH3 in place of a CERV promoter fragment (pNH4). The 729 base pair pea ribulose bisphosphate small subunit terminator [TRBCS] was amplified by PCR with primers TRBSC5 and TRBSC3N using 25 thermal cycles (30 s. 94 °C, 30 s. 53 °C, 120 s. 72 °C) and a mixture of *Thermus aquaticus* (Taq) and Pfu DNA polymerase (9:1). The amplification product was purified using the Qiagen PCR product purification kit. This fragment was digested with *SacI* and *EcoRI* and inserted into pNH4 in place of the nopaline synthase terminator rendering pNH5. Several pNH5 clones were identified by restriction enzyme digestion analysis using *SacI and EcoRI*. These clones all exhibited the characteristic DNA fragment pattern, i. e. 631 and 3509 base pair fragments, when separated in an agarose gel. One of the positive clones was sequenced using primers 35S and U19 (Figure 9 A) on an automatic Perkin Elmer 373 sequencer using dyed fluorescent nucleotides according to the supplier's recommendations. The sequencing confirmed that the TRBCS fragment was correctly amplified. Moreover, sequencing also confirmed that the polylinker region, holding sites *NcoI*, *NheI*, *MunI* and *SacI*, was intact. A cloning scheme covering these steps is shown in Figure 6.

Table 3. Oligonucleotides used in vector construction (given in 5' to 3' direction)

Primer	Sequence
Sal1	AAT TCG CTG GTG TCG ACT TTA CTT
Sal2	CTA GAA GTA AGG TCG ACA CCA GCG
Xma1	AGC TTA CTC TTC CCG GGA TTG TTA T
Xma2	CGA TAA CAA TCC CGG GAA GAG TA
HMGR5	ATA TTT TTC CAT GGA CAC CAC C
HMGR3	GGA CCG AAT TCC CAC TAA GAT GC
TRBCS5	GGA ATG AGC TCT AAA GAG CTA GAG CTT TCG TTC
TRBCS3N	GTC AAT GAA TTC GCA AGT CAT AAA ATG
U19	TTT CCC AGT CAC GAC GTT GT
HMGRisF	GGA TCC CAA CTA CCT CAT
HMGRisR	TCC ACC CAA AGC ACC AG
ISHMGR5	CTG TTC CAA TGG CGA CC
35S	TCC ACT GAC GTA AGG GAT GAC
F72	GCC ATA ATA CTC GAA CTC AG

A 1727 base pair gene fragment encoding the *Hevea brasiliensis hmg* 1 was amplified by PCR from a cDNA clone in order to introduce cloning sites in either end of the gene (accession number X54659, Chye *et al.*, 1991). The *hmg1* cDNA was amplified by gene specific primers (HMGR5 and HMGR3) using 25 thermal cycles (30 s. 94 °C, 30 s. 53 °C, 120 s. 72 °C) and the proof reading enzyme *Pyrococcus furiosus* (Pfu) DNA polymerase to enhance the fidelity. The obtained fragment was digested by *NcoI* and *Eco*RI and inserted between the *NcoI* and *MunI* sites of pNH5 yielding pNH8 (Figure 8). Six pNH8 clones were identified based on

restriction enzyme digestion pattern. These clones displayed 2 fragments of 2378 and 3487 base pairs when digested by *Nco*I and *Eco*RI. Two independent positives clones were chosen for sequencing using the primers shown in Figure 9 B. In both clones the *hmg* 1 genes contained five identical nucleotide substitutions as compared to the published sequence (X54659) (Figure 10). Furthermore, when sequencing the obtained cDNA clone, which had previously been used as the template to amplify the *hmg* 1 gene, it also contained the same five nucleotide substitutions. The codon changes due to the nucleotide substitutions did not give rise to amino acid substitutions, i. e. all nucleotide substitutions were silent mutations. Hence it was concluded that the most probable explanation for these nucleotide substitutions are sequencing errors when the clone was initially cloned and deposited in the gene bank. This conclusion is supported by the fact that all substitutions are confined to a 225 base pairs region in the central part of the *hmg* 1 gene.

Plasmid pNH8 was digested by *Hind*III and *Eco*RI to obtain the 3158 base pair 2x35S-hmgr1-TRBCS cassette which was subsequently inserted into the binary vector pSJ34 rendering pNH16 (Figure 7). The steps of constructing pNH16 are schematically drawn in Figure 8.

Positive pNH16 clones were selected based on restriction enzyme digestion analysis. Clones exhibiting the correct pattern when digested by *Hind*III and *Eco*RI, i. e. 3183 and 11106 base pair fragments, were selected. One of the positive clones was sequenced as shown in Figure 9 C. This confirmed that 5′ and 3′ parts of the *hmg* 1 gene were

correctly fused to the 2x35S promoter and the TRBSC terminator, respectively.

Vectors pNH16 and pSJ34 (vector control) were transformed into *Agrobacterium* LBA4404 using electroporation according to the method of Wen-Jun and Forde (1989). Transformants were analysed for presence of the gene of interest by PCR. Transformation of tobacco was carried out as described in Example 1. As well as the vector control plants a number of untransformed tobacco plants were generated via tissue culture.

Sterol levels were determined in accordance to example 2.

Table 4 shows the sterol analysis of mature seeds obtained from tobacco transformed with the *Hevea brasiliensis* hmg 1 gene fragment under control of the 35S promoter. Seeds from 23 independent transgenic plants (NH16) were analysed along with seeds of 12 independent untransformed plants (SR1) which had been generated via tissue culture.

The total sterol content of the SR1 had a mean of 0.337 % dry weight (S.D.0.019). The HMGR seeds contained total sterol levels of up to 0.389 % dry weight which corresponds to increases of up to 15 % compared to the mean of control seeds.

Table 4: Sterol Analysis of seed from tobacco transformed with 35S - Hevea HMGR (NH16)

Total sterols as % of dry wt

Sample	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Isofucosterol	Cycloartenol	Total sterols
NH16 18	0.0257	0.0545	0.0375	0.1665	0.0723	0.0327	0.389
NH16 21	0.0272	0.0509	0.0356	0.1681	0.0754	0.0275	0.385
NH16 37	0.0293	0.0536	0.0427	0.1589	0.0714	0.0263	0.382
NH16 31	0.0287	0.0485	0.0317	0.1556	0.0749	0.0350	0.374
NH16 28	0.0307	0.0483	0.0340	0.1553	0.0735	0.0265	0.368
NH16 1	0.0266	0.0500	0.0322	0.1432	0.0727	0.0395	0.364
NH16 47	0.0294	0.0459	0.0374	0.1578	0.0710	0.0221	0.364
NH16 23	0.0245	0.0515	0.0368	0.1517	0.0671	0.0301	0.362
NH16 48	0.0268	0.0476	0.0352	0.1518	0.0660	0.0292	0.357
NH16 46	0.0317	0.0469	0.0410	0.1493	0.0595	0.0245	0.353
NH16 12	0.0215	0.0478	0.0443	0.1594	0.0581	0.0212	0.352
NH16 14	0.0248	0.0474	0.0376	0.1528	0.0661	0.0231	0.352
NH16 22	0.0289	0.0478	0.0347	0.1436-	0.0687	0.0275	0.351
NH16 45	0.0220	0.0474	0.0406	0.1595	0.0598	0.0214	0.351
NH16 32	0.0231	0.0497	0.0363	0.1461	0.0626	0.0285	0.346
NH16 19	0.0221	0.0491	0.0395	0.1407	0.0614	0.0297	0.342
NH16 13	0.0218	0.0502	0.0340	0.1420	0.0636	0.0303	0.342
NH16 42	0.0249	0.0467	0.0347	0.1438	0.0630	0.0257	0.339
NH16 27	0.0257	0.0458	0.0339	0.1445	0.0665	0.0183	0.334
NH16 10	0.0262	0.0415	0.0308	0.1451	0.0650	0.0220	0.331
NH16 44	0.0300	0.0436	0.0413	0.1446	0.0536	0.0171	0.330
NH16 3	0.0221	0.0467	0.0373	0.1459	0.0580	0.0178	0.328
NH16 40	0.0270	0.0450	0.0337	0.1338	0.0633	0.0221	0.325
SR1 18(control)	0.0268	0.0497	0.0325	0.1533	0.0766	0.0330	0.372
SR1 6(control)	0.0314	0.0497	0.0347	0.1416	0.0684	0.0337	0.359
SR1 3(control)	0.0290	0.0466	0.0317	0.1427	0.0725	0.0306	0.353
SR1 17(control)	0.0244	0.0459	0.0305	0.1471	0.0678	0.0346	0.350
SR1 2(control)	0.0267	0.0489	0.0400	0.1391	0.0627	0.0212	0.339
SR1 1(control)	0.0271	0.0449	0.0329	0.1357	0.0654	0.0310	0.337
SR1 9(control)	0.0235	0.0459	0.0312	0.1391	0.0681	0.0292	0.337
SR1 7(control)	0.0243	0.0468	0.0365	0.1334	0.0647	0.0305	0.336
SR1 8(control)	0.0274	0.0427	0.0284	0.1261	0.0627	0.0334	0.321
SR1 5(control)	0.0226	0.0442	0.0413	0.1413	0.0547	0.0125	0.317
SR1 4(control)	0.0220	0.0431	0.0367	0.1357	0.0599	0.0176	0.315
SR1 20(control)	0.0160	0.0427	0.0407	0.1346	0.0495	0.0246	0.308

Example 5 Transformation of tobacco with a truncated *Hevea brasiliensis* HMG 1 gene

A truncated form of *Hevea brasiliensis* (H.B.K.) Müll. Arg. *tHMG1*, encoding the enzyme lacking the N-terminal membrane-binding domain, was cloned using the primers based on the published sequence Chye *et al.*, 1991. The forward primer

5'-CCTACCTCGGAAGCC**ATG**GTTGCAC-3' incorporates a new start codon (bold) and a *Nco I* restriction site (underlined) for cloning applications. The reverse primer 5'-CATTTTACATTGCTAGCACCAGATTC-3' contains a *Nhe I* restriction site (underlined) for downstream sub-cloning purposes. The plasmid pNH8 (Figure 8) was used as the template DNA in the PCR (30 cycles) using *Pfu* polymerase under standard conditions and produced a fragment of the expected size ~1.3 kb. The resulting *thmg1* gene (Figure 11 a) codes for amino acids 153-575 of the full-length (575) *hmg1* sequence (Figure 11 b). The PCR product was cloned into the pGEM-T vector (Promega) according to the manufacturers instructions and sequenced to confirm correct sequence.

The *H. brasiliensis thmg1* was inserted into pNH4 (Fig.6) between the *Nco I* and *Nhe I* sites of the polylinker, which lie between the CaMV 35S double promoter and nos terminator to give pMH3 (Figure 13). This chimeric gene was isolated by digestion with *Xma CI* and *Sal I*, purified and cloned into the corresponding polylinker sites in pSJ34 (Figure 5), this binary construct was named MH3 (Figure 15). MH3 was sequenced to check that the *hmg1* genes had been inserted correctly and there were no mistakes in the promoter-initiation and terminator sequences. Vectors MH5 and pSJ34 (vector control) were then transferred into *A. tumefaciens* strain LBA4404 by electroporation. Transformation of tobacco was carried out as described in Example 1.

Sterol levels in leaf and seeds were determined in accordance to example 2, but with the following modifications. After extraction and transmethylation,

sterols are dissolved in 250-300µl of toluene and silyated by the addition of 125-150µl of 95:5 N,O-bis(trimethylsilyl)acetamide:trimethylchlorosilane followed by incubation at 50°C for 10 minutes. GC analysis is carried out using a 25 m x 0.32 mm i.d. (0.25 µm film thickness) 5% BPX5 column (ex SGE) in a Perkin-Elmer Autosystem XL GC. The temperature program is 80-230 at 45 °C/min, 230-280 at 4 °C/min, 280-355 at 20 °C/min, and 5 min. at 355 °C. The FID temperature is 370 °C, the helium pressure 8 psi, the injection volume 1.0 µL and the split flow 10 mL/min. A GC response factor of 1.0 for each of the sterols with respect to the dihydrocholesterol internal calibrant is assumed. This method afforded improved separation of sterol intermediate compounds. As a result, in addition to the sterol compounds identified in Example 2, Δ-7-avenasterol, squalene, 24-methylene cycloartanol, 24-methylene lophenol and 24-ethylidene lophenol were also identified by comparison with authentic samples, library spectra and literature data following GC-MS analysis as described in Example 2.

Table 5 shows the sterol analysis of leaves from 29 independent transgenic plants (MH5) and five untransformed control plants (SR1). The average total sterol content of the SR1 leaves was 0.180% dry weight (S.D.=0.017), whereas the sterol content of the MH5 leaves ranged from 0.189 - 1.931% dry weight. The MH5 figures correspond to increases in total sterol content of up to 10.7-fold over the control mean.

Table 6 shows the sterol analysis of mature seeds from 27 independent transgenic plants (MH5) and 8 SR1 untransformed

control plants. The average total sterol content of the SR1 seeds was 0.368% dry weight (S.D.=0.039), whereas the total sterol content of the MH5 seeds ranged from 0.352-0.874% dry weight. The MH5 figures correspond to increases of up to 2.4-fold in total sterol and 1.7-fold in 4-desmethylsterol levels over the respective control means.

Further analysis of MH5 33 seed was carried out to determine the proportion of free and esterified sterol in the sample. The total lipid fraction is isolated as described in Example 2, but not subjected to the transmethylation process. The lipid residue, which contains dihydrocholesterol as internal standard, is dissolved in 40-60 petroleum ether (250 µL) and applied to a glass-backed 20 cm x 20 cm x 0.5 mm silica gel thin layer chromatography (TLC) plate. The vial that contained the lipid residue is washed out with a further 250 µL aliquot of petroleum ether, which is also applied to the plate. A 10 µL aliquot of a solution consisting of a mixture of ß-sitosterol (10 mg) and cholesterol oleate (10 mg) dissolved in acetone (1 mL) is spotted to act as a marker. The plate is developed using 60-80 petroleum ether-diethyl ether-acetic acid (80:20:2, v/v/v). The sterol fractions are visualised by spraying with a 0.01 % w/v ethanolic solution of rhodamine 6G and viewing the plate under UV light. Approximate R_f values are 0.25 for free sterols and 0.9 for steryl esters. The free sterol band is scraped off the plate and transferred to a vial. The free sterol fraction is isolated by washing the band with three volumes of diethyl ether. The ether washings are combined and filtered. The free sterol fraction, isolated by blowing off

the solvent with nitrogen gas, is silylated and analysed by gas chromatography (GC) as described in Example 2. Amounts of esterified sterol are determined by subtracting amounts of free sterol from total sterol.

Table 7 shows the analyses of the free sterol and sterol ester fractions of transgenic MH5 seed samples 6 and 33, alongside that of an SR1 control sample. The additional sterol present in the transgenic samples compared to the control is found primarily in the form of sterol esters. The total sterol content of the SR1 control is 0.388% dry weight, of which 52.4% is in the form of esters. The total sterol contents of MH5 6 and 33 are 0.711% and 0.866% dry weight respectively, of which 68.8% and 74.2% respectively are esterified.

Table 5

Ster l analysis of leaf from tobacco transformed with 35S- truncated Hevea HMGR (MH5)

Total sterols as % of dry wt

Smpl code	squalene	cycloartenol	24 methylene cycloartanol	24 methylene lophenol	24 ethylidene lophenol	d7 avenasterol	isofucosterol	sitosterol	stigmasterol	campesterol	cholesterol	Total
MH5 55	0.1623	0.9066	0.0583	0.0902	0.0829	0.0290	0.1483	0.1552	0.0994	0.0698	0.1285	1.931
MH5 23	0.1387	0.5640	0.0495	0.0779	0.0930	0.0154	0.1498	0.1554	0.1020	0.0680	0.0321	1.446
MH5 53	0.0955	0.5420	0.0482	0.0753	0.1040	0.0198	0.1593	0.1455	0.1162	0.0709	0.0424	1.419
MH5 18	0.1036	0.6315	0.0396	0.0696	0.0658	0.0097	0.1340	0.1294	0.0930	0.0617	0.0526	1.391
MH5 32	0.1419	0.6040	0.0276	0.0640	0.0687	0.0156	0.1342	0.1035	0.0797	0.0579	0.0536	1.351
MH5 44	0.0443	0.6869	0.0374	0.0563	0.0755	0.0167	0.1541	0.1129	0.0844	0.0497	0.0104	1.329
MH5 15	0.0950	0.5648	0.0357	0.0644	0.0746	0.0169	0.1579	0.1190	0.0917	0.0578	0.0223	1.300
MH5 51	0.0756	0.5796	0.0336	0.0536	0.0682	0.0146	0.1302	0.1167	0.0949	0.0507	0.0234	1.241
MH5 2	0.0362	0.4601	0.0412	0.0686	0.0633	0.0126	0.0929	0.1510	0.1059	0.0803	0.0407	1.153
MH5 40	0.0431	0.5741	0.0295	0.0488	0.0506	0.0141	0.1128	0.0932	0.0730	0.0466	0.0361	1.122
MH5 30	0.0782	0.4141	0.0208	0.0530	0.0732	0.0148	0.1281	0.1318	0.0985	0.0511	0.0220	1.086
MH5 33	0.0332	0.5526	0.0314	0.0457	0.0456	0.0146	0.1118	0.0831	0.0728	0.0454	0.0320	1.068
MH5 13	0.0692	0.4583	0.0348	0.0441	0.0543	0.0080	0.1226	0.0976	0.0874	0.0432	0.0310	1.050
MH5 12	0.0510	0.3312	0.0355	0.0618	0.0531	0.0070	0.0888	0.1254	0.1028	0.0765	0.0268	0.960
MH5 6	0.0366	0.3859	0.0309	0.0536	0.0608	0.0135	0.1015	0.1092	0.0986	0.0633	0.0059	0.960
MH5 37	0.0428	0.3941	0.0208	0.0447	0.0503	0.0109	0.1143	0.0936	0.0869	0.0687	0.0190	0.946
MH5 25	0.0259	0.3674	0.0264	0.0474	0.0519	0.0071	0.0988	0.1113	0.0949	0.0466	0.0208	0.899
MH5 21	0.0110	0.2794	0.0267	0.0487	0.0550	0.0084	0.1091	0.1291	0.1078	0.0844	0.0197	0.879
MH5 54	0.0033	0.1510	0.0206	0.0353	0.0403	0.0076	0.0903	0.1075	0.0979	0.0823	0.0272	0.663
MH5 17	0.0083	0.1516	0.0224	0.0319	0.0224	0.0045	0.0677	0.0788	0.0976	0.0700	0.0212	0.576
MH5 26	0.0049	0.1443	0.0162	0.0113	0.0241	0.0062	0.0552	0.0862	0.1098	0.0613	0.0199	0.539
MH5 35	0.0000	0.0373	0.0147	0.0144	0.0035	0.0021	0.0161	0.0276	0.0954	0.0459	0.0265	0.284
MH5 42	0.0000	0.0208	0.0094	0.0083	0.0055	0.0026	0.0112	0.0345	0.1171	0.0513	0.0160	0.277
MH5 31	0.0000	0.0219	0.0093	0.0054	0.0039	0.0024	0.0241	0.0470	0.0952	0.0424	0.0189	0.271
MH5 14	0.0012	0.0167	0.0069	0.0189	0.0049	0.0021	0.0192	0.0320	0.1023	0.0453	0.0190	0.268

MH5 22	0.0000	0.0117	0.0112	0.0117	0.0035	0.0023	0.0282	0.0281	0.0922	0.0440	0.0151	0.248
MH5 43	0.0000	0.0119	0.0065	0.0057	0.0029	0.0030	0.0192	0.0244	0.0834	0.0460	0.0152	0.218
MH5 46	0.0000	0.0043	0.0040	0.0025	0.0033	0.0030	0.0087	0.0349	0.0951	0.0450	0.0116	0.212
MH5 8	0.0000	0.0090	0.0056	0.0035	0.0032	0.0029	0.0172	0.0252	0.0735	0.0362	0.0131	0.189
SR1 6	0.0000	0.0065	0.0047	0.0046	0.0038	0.0018	0.0116	0.0265	0.0859	0.0371	0.0186	0.201
SR1 1	0.0000	0.0101	0.0042	0.0036	0.0023	0.0020	0.0124	0.0233	0.0839	0.0374	0.0172	0.196
SR1 9	0.0000	0.0033	0.0021	0.0000	0.0014	0.0026	0.0174	0.0233	0.0748	0.0354	0.0127	0.173
SR1 8	0.0000	0.0183	0.0054	0.0053	0.0024	0.0017	0.0123	0.0211	0.0623	0.0286	0.0138	0.171
SR1 10	0.0000	0.0033	0.0029	0.0025	0.0017	0.0024	0.0135	0.0183	0.0647	0.0361	0.0115	0.157

Table 6

Sterol analysis of seed from tobacco transformed with 35S-truncated Hevea HMGR (MH5)

Total sterols as % of dry wt

Smplcode	squalene	cycloartenol	24 methylene cycloartanol	24 methylene lophenol	24 ethylidene lophenol	d7 avena sterol	isofucosterol	sitosterol	stigmasterol	campesterol	cholesterol	Total
MH5 33	0.0084	0.2582	0.0444	0.0250	0.0419	0.0129	0.1272	0.1915	0.0612	0.0801	0.0234	0.874
MH5 22	0.0158	0.1324	0.0152	0.0178	0.0660	0.0088	0.1288	0.2019	0.0349	0.0655	0.0368	0.724
MH5 6	0.0112	0.1482	0.0358	0.0202	0.0508	0.0066	0.1184	0.1954	0.0430	0.0659	0.0273	0.723
MH5 15	0.0087	0.1578	0.0397	0.0209	0.0348	0.0095	0.1029	0.1837	0.0639	0.0760	0.0243	0.722
MH5 5	0.0039	0.1965	0.0539	0.0192	0.0286	0.0199	0.0964	0.1437	0.0620	0.0648	0.0136	0.703
MH5 23	0.0112	0.1335	0.0269	0.0174	0.0447	0.0092	0.1209	0.1769	0.0377	0.0598	0.0344	0.673
MH5 55	0.0143	0.1425	0.0275	0.0188	0.0438	0.0071	0.1060	0.1815	0.0402	0.0599	0.0304	0.672
MH5 35	0.0140	0.0785	0.0103	0.0147	0.0888	0.0090	0.1181	0.2080	0.0360	0.0595	0.0322	0.669
MH5 37	0.0132	0.1328	0.0265	0.0152	0.0414	0.0093	0.1176	0.1676	0.0352	0.0534	0.0349	0.647
MH5 25	0.0152	0.1217	0.0257	0.0159	0.0467	0.0060	0.1028	0.1747	0.0356	0.0495	0.0315	0.626
MH5 2	0.0056	0.0819	0.0121	0.0177	0.0513	0.0125	0.1067	0.2010	0.0438	0.0608	0.0214	0.615
MH5 21	0.0076	0.0710	0.0210	0.0164	0.0451	0.0087	0.1113	0.1776	0.0381	0.0584	0.0387	0.594
MH5 42	0.0101	0.1063	0.0232	0.0149	0.0438	0.0049	0.1033	0.1650	0.0350	0.0494	0.0328	0.589
MH5 53	0.0095	0.1026	0.0303	0.0148	0.0393	0.0045	0.0960	0.1716	0.0363	0.0571	0.0257	0.588
MH5 13	0.0053	0.1039	0.0269	0.0164	0.0359	0.0069	0.0980	0.1645	0.0452	0.0530	0.0283	0.584
MH5 51	0.0080	0.0953	0.0289	0.0151	0.0414	0.0086	0.0844	0.1727	0.0419	0.0575	0.0244	0.578
MH5 12	0.0104	0.0619	0.0090	0.0136	0.0486	0.0077	0.1078	0.1799	0.0425	0.0616	0.0297	0.573
MH5 26	0.0117	0.0558	0.0064	0.0137	0.0518	0.0065	0.1038	0.1793	0.0362	0.0602	0.0324	0.558
MH5 18	0.0110	0.0835	0.0174	0.0151	0.0431	0.0063	0.0930	0.1634	0.0385	0.0565	0.0281	0.556
MH5 54	0.0105	0.0380	0.0078	0.0073	0.0376	0.0047	0.0781	0.1470	0.0327	0.0480	0.0381	0.450
MH5 17	0.0079	0.0398	0.0086	0.0109	0.0360	0.0044	0.0718	0.1467	0.0348	0.0467	0.0254	0.433
MH5 14(1)	0.0056	0.0301	0.0041	0.0058	0.0331	0.0038	0.0585	0.1493	0.0388	0.0467	0.0205	0.396
MH5 14(2)	0.0062	0.0318	0.0025	0.0052	0.0340	0.0041	0.0623	0.1456	0.0355	0.0446	0.0232	0.395
MH5 8	0.0063	0.0306	0.0039	0.0058	0.0307	0.0042	0.0673	0.1377	0.0330	0.0471	0.0255	0.392
MH5 43	0.0074	0.0311	0.0026	0.0063	0.0335	0.0037	0.0634	0.1360	0.0323	0.0439	0.0243	0.385

MH5 40	0.0059	0.0309	0.0029	0.0068	0.0330	0.0036	0.0655	0.1347	0.0303	0.0428	0.0255	0.382
MH5 10	0.0047	0.0245	0.0040	0.0060	0.0240	0.0038	0.0557	0.1323	0.0389	0.0454	0.0219	0.361
MH5 46	0.0070	0.0276	0.0020	0.0048	0.0260	0.0028	0.0588	0.1288	0.0297	0.0402	0.0245	0.352
SR1 10	0.0070	0.0320	0.0028	0.0062	0.0355	0.0042	0.0689	0.1434	0.0344	0.0487	0.0249	0.408
SR1 4	0.0084	0.0336	0.0029	0.0057	0.0356	0.0038	0.0652	0.1398	0.0325	0.0460	0.0251	0.398
SR1 6	0.0084	0.0296	0.0025	0.0058	0.0362	0.0037	0.0694	0.1420	0.0301	0.0442	0.0261	0.398
SR1 5	0.0069	0.0359	0.0028	0.0056	0.0340	0.0035	0.0646	0.1370	0.0299	0.0457	0.0233	0.389
SR1 3	0.0080	0.0289	0.0028	0.0055	0.0336	0.0031	0.0616	0.1312	0.0314	0.0417	0.0225	0.370
SR1 1	0.0064	0.0288	0.0034	0.0053	0.0302	0.0032	0.0614	0.1351	0.0297	0.0395	0.0255	0.368
SR1 7	0.0023	0.0187	0.0024	0.0014	0.0228	0.0035	0.0422	0.1241	0.0405	0.0412	0.0156	0.315
SR1 8	0.0025	0.0145	0.0016	0.0013	0.0199	0.0024	0.0421	0.1175	0.0374	0.0387	0.0168	0.295

Table 7

Analysis of free sterol and sterol ester fractions of MH5 transgenic seed samples

Sterols as % of dry wt

Sample/Fraction	cycloartenol	24 methylene cycloartanol	24 methylene lophenol	24 ethylidene lophenol	d7 avena sterol	isofucosterol	sitosterol	stigmasterol	campesterol	cholesterol	**Total**
SR1 control											
Total sterol (TS)	0.0260	0.0161	0.0000	0.0237	0.0017	0.0534	0.1615	0.0366	0.0486	0.0205	**0.388**
Free Sterol (FS)	0.0126	0.0032	0.0000	0.0156	0.0000	0.0191	0.0726	0.0314	0.0244	0.0060	0.185
Sterol Ester (=TS-FS)	0.0134	0.0129	0.0000	0.0081	0.0017	0.0343	0.0889	0.0052	0.0241	0.0145	0.203
MH5 6											
Total sterol (TS)	0.1482	0.0358	0.0202	0.0508	0.0066	0.1184	0.1953	0.0429	0.0659	0.0272	**0.711**
Free sterol (FS)	0.0207	0.0114	0.0046	0.0217	0.0017	0.0306	0.0786	0.0260	0.0201	0.0067	0.222
Sterol Ester (=TS-FS)	0.1275	0.0244	0.0156	0.0291	0.0049	0.0878	0.1167	0.0169	0.0458	0.0205	0.489
MH5 33											
Total Sterol (TS)	0.2582	0.0444	0.0250	0.0419	0.0129	0.1272	0.1915	0.0612	0.0801	0.0234	**0.866**
Free Sterol (FS)	0.0215	0.0181	0.0025	0.0104	0.0022	0.0276	0.0717	0.0363	0.0256	0.0072	0.223
Sterol Ester (=TS-FS)	0.2367	0.0263	0.0226	0.0315	0.0107	0.0996	0.1198	0.0249	0.0545	0.0162	0.643

% FS vs. SE for sterol components															
SR1 control															
Free Sterol	48.6	19.9		0.0		65.9		0.0		35.8		45.0		85.7		50.3		29.1		47.6
Sterol Ester	51.4	80.1		0.0		34.1		100.0		64.2		55.0		14.3		49.7		70.9		52.4
MH5 6																				
Free sterol	14	32		23		42.6		25.2		25.8		40.3		60.6		30.5		24.7		31.2
Sterol ester	86	68		77		57.4		74.8		74.2		59.7		39.4		69.5		75.3		68.8
MH5 33																				
Free sterol	8.3	40.8		9.9		24.7		16.9		21.7		37.4		59.3		31.9		30.6		25.8
Sterol Ester	91.7	59.2		90.1		75.3		83.1		78.3		62.6		40.7		68.1		69.4		74.2

Example 6 Transformation of tobacco with a truncated *S.cerevisae* HMGR gene

Based on the nucleotide sequence of cosmid 8248 from the *Saccharomyces cerevisae* chromosome XIII sequencing project, primers were designed to clone the *tHMG1* gene by polymerase chain reaction. The forward primer 5'-GCTTGGATAAGGCC**ATG**GGTCCTTTAG-3' incorporates a new start codon (bold) and a *Nco I* restriction site (underlined) for cloning purposes. The reverse primer 5'-GAATACCAATGAGCTCTGACTAAG-3' contains a *Sac I* restriction site (underlined) for sub-cloning applications. Prior to PCR the genomic DNA from *S. cerevisae*, NCYC 957, X2180, α, SUC2, mal, gal2, CUA was digested with *Eco RI* and the DNA fractionated on a 0.7 % agarose gel. DNA fragments ~2.0 kb in size were excised from the gel and purified using the Qiagen QIAquick gel extraction kit, according to the manufacturers protocol. This DNA was used as the template in the subsequent PCR. The PCR (35 cycles) was performed using *Taq* and *Pfu* polymerase (3:1) under standard conditions and produced a DNA fragment of the expected size ~1.4 kb. The resulting *tHMG1* gene (Figure 12a) codes for amino acids 598-1054 of the full length (1054) *HMG1* sequence (Figure 12 b). The *tHMG1* PCR product was cloned into the pGEM-T vector (Promega) according to the manufacturers instructions and sequenced to confirm the correct sequence. The *S. cerevisae tHMG1* was inserted into pNH4 (Figure 6) between the *Nco I* and *Sac I* sites of the polylinker to produce pMH4 (Figure 14). This chimaeric gene was isolated by digestion with *Xma CI* and *Sal I*, purified and cloned into the corresponding polylinker

sites in pSJ34 (Figure 5), to create the binary plasmid pMH6 (Figure 16). pMH4 was sequenced to check that the *HMG1* gene had been inserted correctly and there were no mistakes in the promoter-initiation and terminator sequences. Vectors MH6 and pSJ34 (vector control) were then transferred into *A. tumefaciens* strain LBA4404 by electroporation. Transformation of tobacco was carried out as described in Example 1.

Seeds were analysed in accordance to Example 5. The results showing (see table 8) an increase in total sterol levels of the transgenic plants (MH 6) of up to 16 % compared to the mean of the control plants (SR1, mean 0.373).

Table 8

Ster 1 analysis of seed from tobacco transformed with 35S- truncated S. cerevisiae HMGR (MH6)

Total sterols as % of dry wt

Smplcode	squalene	cycloartenol	24 methylene cycloartanol	24 methylene lophenol	24 ethylidene lophenol	d7 avenasterol	isofucosterol	sitosterol	stigmasterol	campesterol	cholesterol	Total
MH6 6	0.0075	0.0415	0.0064	0.0040	0.0395	0.0071	0.0666	0.1507	0.0352	0.0486	0.0258	0.433
MH6 33	0.0067	0.0320	0.0061	0.0053	0.0356	0.0071	0.0746	0.1275	0.0346	0.0445	0.0442	0.418
MH6 7	0.0058	0.0310	0.0045	0.0055	0.0325	0.0064	0.0687	0.1415	0.0393	0.0497	0.0270	0.412
MH6 22	0.0065	0.0337	0.0058	0.0053	0.0352	0.0062	0.0644	0.1481	0.0350	0.0479	0.0233	0.412
MH6 18	0.0077	0.0328	0.0047	0.0052	0.0360	0.0064	0.0663	0.1395	0.0335	0.0462	0.0279	0.406
MH6 38	0.0055	0.0246	0.0046	0.0047	0.0288	0.0057	0.0720	0.1423	0.0351	0.0510	0.0290	0.403
MH6 1	0.0053	0.0260	0.0044	0.0050	0.0303	0.0060	0.0631	0.1432	0.0404	0.0508	0.0251	0.400
MH6 8	0.0063	0.0262	0.0058	0.0050	0.0316	0.0058	0.0677	0.1380	0.0339	0.0477	0.0299	0.398
MH6 26	0.0065	0.0351	0.0048	0.0052	0.0332	0.0063	0.0580	0.1379	0.0347	0.0455	0.0252	0.392
MH6 34	0.0064	0.0341	0.0042	0.0039	0.0299	0.0061	0.0624	0.1325	0.0382	0.0459	0.0276	0.391
MH6 10	0.0048	0.0280	0.0060	0.0043	0.0298	0.0049	0.0579	0.1481	0.0378	0.0479	0.0218	0.391
MH6 23	0.0054	0.0288	0.0058	0.0050	0.0268	0.0055	0.0643	0.1334	0.0369	0.0499	0.0258	0.387
MH6 20	0.0049	0.0345	0.0055	0.0039	0.0322	0.0066	0.0555	0.1442	0.0357	0.0418	0.0213	0.386
MH6 31	0.0050	0.0326	0.0037	0.0045	0.0323	0.0062	0.0566	0.1404	0.0322	0.0423	0.0210	0.377
MH6 29	0.0044	0.0239	0.0030	0.0046	0.0240	0.0059	0.0572	0.1306	0.0355	0.0468	0.0264	0.362
MH6 28	0.0058	0.0220	0.0046	0.0042	0.0223	0.0046	0.0559	0.1249	0.0425	0.0501	0.0232	0.360
MH6 15	0.0044	0.0252	0.0043	0.0042	0.0226	0.0046	0.0528	0.1197	0.0418	0.0476	0.0245	0.352
MH6 9	0.0034	0.0183	0.0034	0.0033	0.0193	0.0044	0.0522	0.1286	0.0449	0.0499	0.0223	0.350
MH6 32	0.0038	0.0216	0.0031	0.0035	0.0244	0.0052	0.0512	0.1247	0.0397	0.0448	0.0225	0.345
MH6 12	0.0027	0.0244	0.0051	0.0035	0.0246	0.0047	0.0439	0.1233	0.0433	0.0458	0.0166	0.338
MH6 37	0.0029	0.0113	0.0029	0.0025	0.0134	0.0025	0.0383	0.1129	0.0464	0.0490	0.0172	0.299
SR1 7	0.0055	0.0280	0.0043	0.0044	0.0296	0.0064	0.0631	0.1377	0.0342	0.0458	0.0263	0.385
SR1 8	0.0058	0.0307	0.0037	0.0045	0.0309	0.0052	0.0594	0.1311	0.0359	0.0471	0.0244	0.379
SR1 1	0.0052	0.0284	0.0035	0.0052	0.0288	0.0057	0.0608	0.1305	0.0368	0.0468	0.0262	0.378
SR1 5	0.0041	0.0206	0.0026	0.0042	0.0239	0.0039	0.0537	0.1291	0.0393	0.0477	0.0231	0.352
Average	0.0052	0.0269	0.0035	0.0046	0.0283	0.0053	0.0593	0.1321	0.0366	0.0468	0.0250	0.373

Example 7 Transformation of tobacco with truncated *Hevea brasiliensis HMGR1* cDNA linked to a seed-specific promoter

The *H. brasiliensis tHMGR1* was also cloned into the polylinker region of pNH12 in the *Nco I* and *Nhe I* restriction sites, which lie between the ACP (acyl-carrier protein) promoter and the nos terminator to give construct pMH11. The chimeric gene was cloned into the binary vector pSJ34 after digestion and purification with *XmaC I* and *EcoR I* and named pMH15. The binary vector pMH15 was sequenced to check that the *hmgr1* gene had been inserted correctly and there were no mistakes in the promoter-initiation and terminator sequences. The binary plasmid was used to transform the *A. tumefaciens* strain LBA4404 by electroporation.

Tobacco was transformed with this plasmid in accordance to example 1.

Example 8 Transformation of *Brassica napus* (oil seed rape) with truncated *Hevea brasiliensis* gene of example 5

Electrocompetent *Agrobacterium tumefaciens* cells (strain LBA4404) were defrosted on ice and 5ng of vector plasmid MH5 (as above) added. Cells plus plasmid were then placed into a pre-chilled electroporation cuvette and electroporated in a Bio Rad Gene Pulser at a capacitance of 25 and at 600 ohms. Immediately after electroporation 950µF of 2X TY broth was added, the cells mixed gently and placed in a sterile vial. The cells were shaken at 28°C for 2 hours and 25µl aliquots plated on solid Lennox media containing rifampicin 50µg/ml and kanamycin 50µg/ml and

incubated at 28°c for 3 days. Single colonies were used to inoculate 10µl of water (for PCR confirmation) and 500µl of Lennox media containing rifampicin 50µg/ml and kanamycin 50µg/ml.

Seeds of B.napus cv.Westar were surface sterilised in 1% sodium hypochlorite for 20 mins. The seeds were washed in sterile distilled water 3 times and plated at a density of 10 seeds per plate on MSMO with 3% sucrose pH 5.8. Seeds were germinated at 24°C in a 16 h light / 8 h dark photoperiod. After 3-4 days, the cotyledons, including 2mm of petiole, were excised. Care was taken to remove the apical meristem and to keep the cotyledon out of the medium. The excised cotyledons were placed on MS medium, 3% sucrose and 0.7% agar with 20 µM 6-benzylaminopurine (BAP). Petioles with attached cotyledons were embedded in this medium to a depth of approximately 2mm at 10 per plate. For transformation, individual excised cotyledons were taken from the plates and the cut surface of their petiole immersed into the agrobacterium suspension for a few seconds. They were then returned to the MS plates and co-cultivated with the agrobacterium for 72 h. After co-cultivation, the cotyledons were transferred to regeneration medium (MS medium with 20µM BAP, 3% sucrose, 0.7% agar, pH 5.8 with 400mg/l augmentin and 15 mg/l kanamycin sulphate). The petioles were, as before, embedded to a depth of 2mm at a density of 10 explants per plate, and again the cotyledon was kept out of the medium. After 2 or 3 weeks, shoots had appeared, some of which bleached by the fourth week, the remaining green shoots were sub-cultured onto shoot elongation medium (regeneration medium

minus BAP). After 1 or 2 weeks, when apical dominance had been established, the shoots were transferred to rooting medium [MS medium, 3% sucrose, 2 mg/l indole butyric acid (IBA), 0.7% agar and 400mg/l augmentin (no kanamycin)]. As soon as a small root mass was obtained, the plantlets were transferred to potting mix supplemented with fertiliser granules. The plants were grown in a misting chamber (average humidity 75%) for 2- 3 weeks at 24°C, 16h light / 8h dark photoperiod. After 3 weeks the plants were transferred to the glasshouse and allowed to flower and set seed.

Claims

1. The use of a gene expressing a non-feed back inhibited HMG-reductase to increase the level of 4-desmethyl sterols in the seeds of plants.

2. The use according to claim 1, wherein the level of 4-desmethylsterols is increased in the seeds by at least 10%.

3. The use according to claim 1, wherein the seeds are oilseeds.

4. The use according to claim 3, wherein the oilseeds are from tobacco, canola, sunflower, rape or soy.

5. The use according to claim 1, wherein the non feedback inhibited HMG-reductase is expressed by a truncated non-plant HMG gene.

6. The use according to claim 5, wherein the HMG-reductase expressed by the truncated HMG gene lacks the membrane binding domain.

7. The use according to claim 1, wherein the non-feedback inhibited HMG-reductase is expressed by a truncated plant HMG gene.

8. The use according to claim 1, wherein the HMG-reductase can be derived from *Asteraceae*.

9. The use according to claim 8, wherein the HMGR gene can de derived from *Hevea brasiliensis* or the HMGR gene is

a truncated version of a gene which can be derived from *Hevea brasiliensis*.

10. Use according to claim 9, wherein the HMGR gene is the hmg 1 gene derived from *Hevea brasiliensis* or a truncated version of said gene.

11. Use of a heterologous gene expressing a truncated non-feed back inhibited HMG-reductase to increase the level of sterols in plants.

12. Use according to claim 11 wherein the heterologous gene is derived from *Hevea brasiliensis*.

13. Method of obtaining seeds by

(a) transforming a plant by:

1. transforming a plant cell with a recombinant DNA construct comprising a DNA segment encoding a polypeptide with non feedback inhibited HMGR activity and a promoter for driving the expression of said polypeptide in said plant cell to form a transformed plant cell.

2. regenerating the transformed plant cell into the transgenic plant.

3. selecting transgenic plants that have enhanced levels of 4-desmethylsterols in the seeds compared to wild type strains of the same plant

(b) cultivating the transformed plant for one or more generations;

(c) harvesting seed from the plant grown under(b).

14. Method of obtaining seeds by
(a) transforming a plant by:
1. transforming a plant cell with a recombinant DNA construct comprising a heterologous plant DNA segment encoding a truncated polypeptide with HMGR activity and a promoter for driving the expression of said polypeptide in said plant cell to form a transformed plant cell.
2. regenerating the transformed plant cell into the transgenic plant.
3. selecting transgenic plants that have enhanced levels of sterols compared to wild type strains of the same plant
(b) cultivating the transformed plant for one or more generations;
(c) harvesting the plant grown under(b).

15. Plant obtainable by a method according to claim 14.

16. Plant tissue obtained from a plant according to claim 15.

17. Plant tissue according to claim 16, selected from the group of leaves, fruit and seeds.

18. Plant having incorporated in its genome a heterologous gene encoding a truncated polypeptide HMGR activity.

19. Plant according to claim 18 wherein the heterologous gene is derived from *Asteraceae*.

20. Plant according to claim 19 wherein the heterologous gene is derived from *Hevea brasiliensis*.

21. Plant according to claim 18-20 wherein the truncated polypeptide lacks the membrane binding domain.

22. Plant according to one or more of claims 18-21 selected from vegetables, oilseeds or fruit-trees.

23. Plant tissue having enhanced levels of sterols and produced by a plant according to one or more of claims 18-21.

24. Plant tissue according to claim 22 selected from the group of leaves, fruits or seeds.

25. Seeds having enhanced level of 4-desmethyl sterols and produced by a plant having non-feedback inhibited HMGR activity.

26. Method of obtaining oil comprising 4-desmethyl sterols by extracting oilseeds in accordance to claim 10 or 11.

27. Food product comprising an oil obtained in accordance to claim 12.

Fig.1.

Vector pHEV36

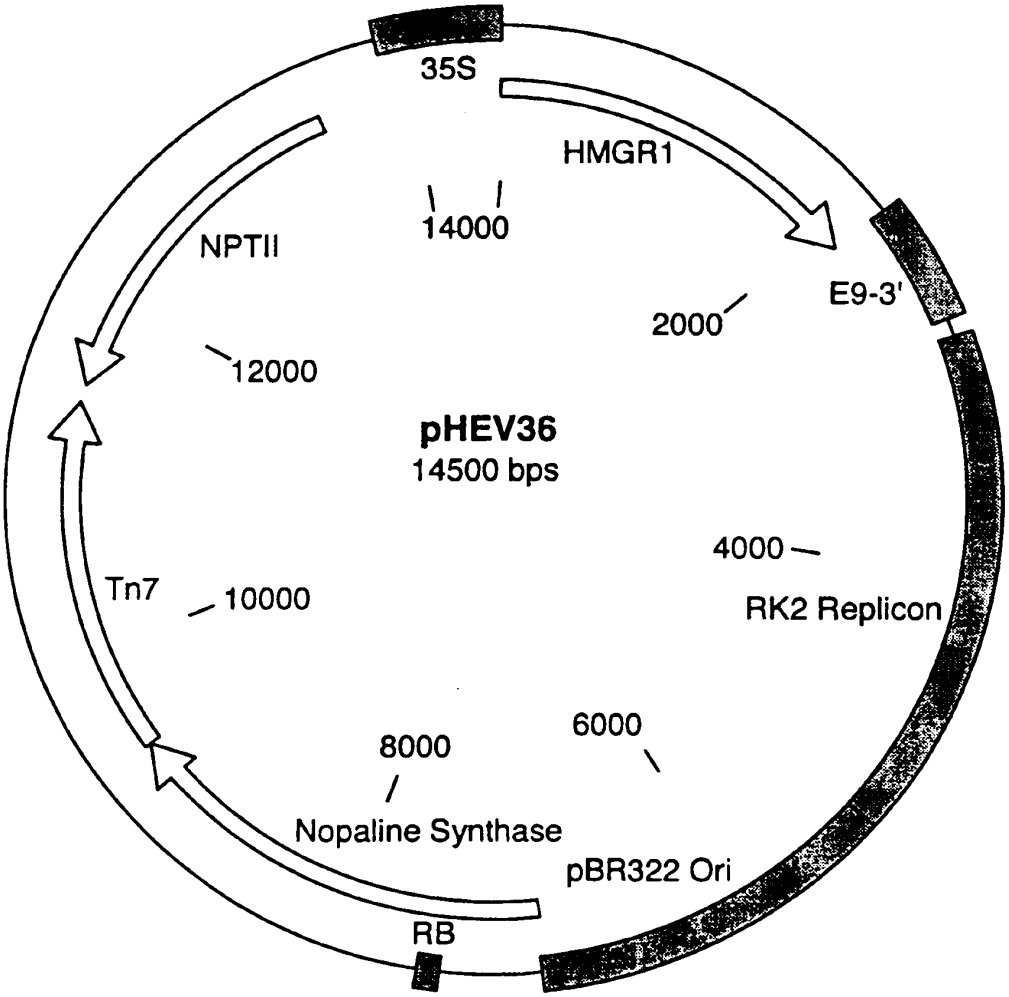


Fig.2.
Vector CJ151

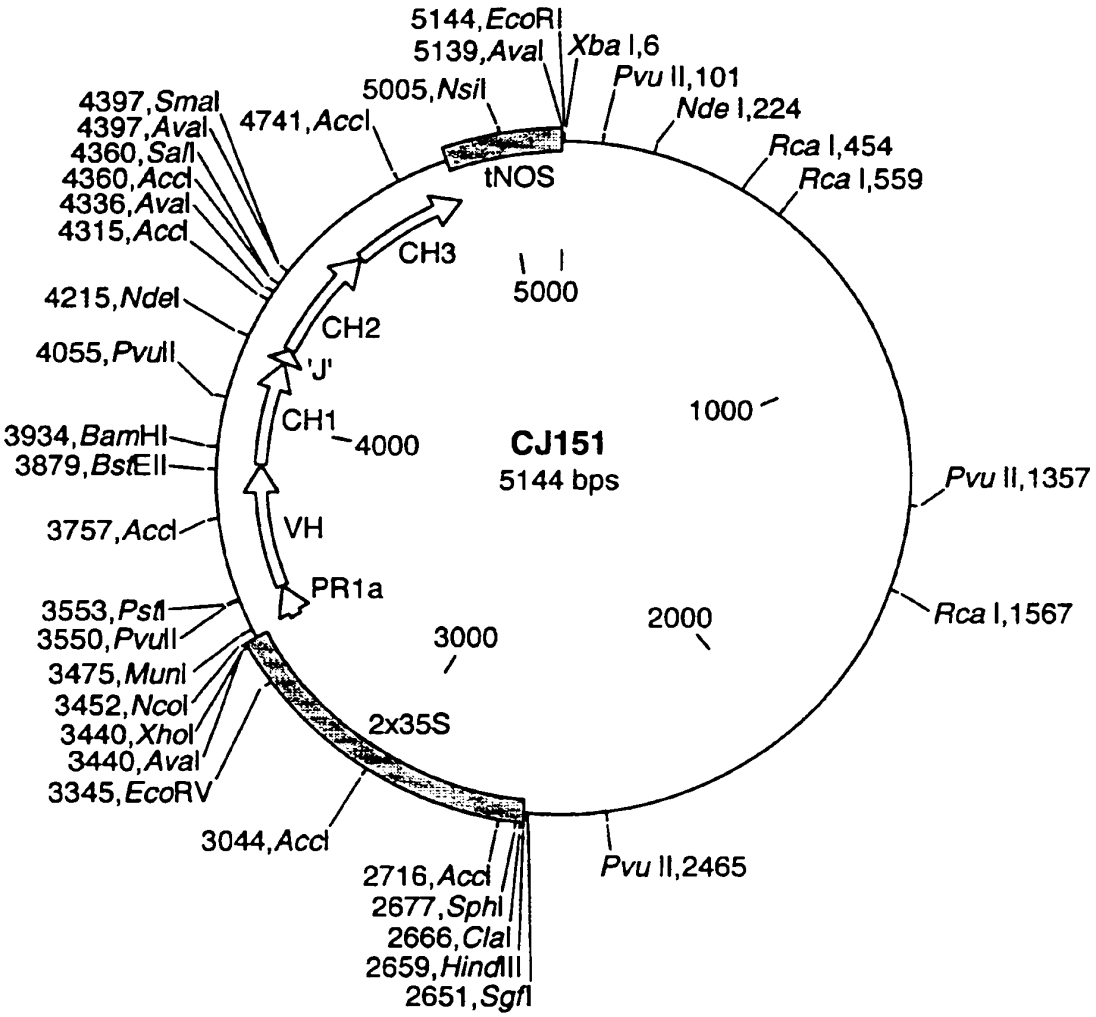


Fig.3.
Vector CJ157

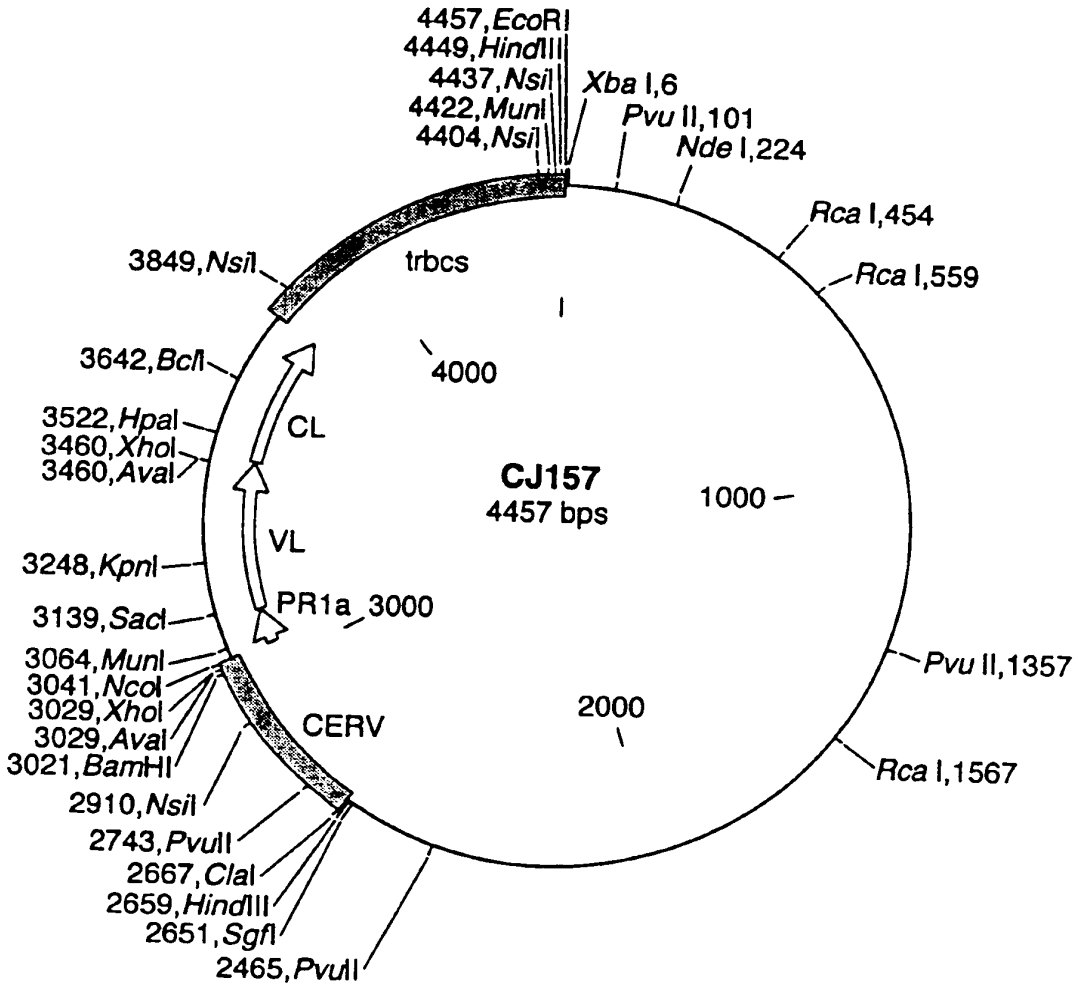


Fig.4.
Vector pp51n

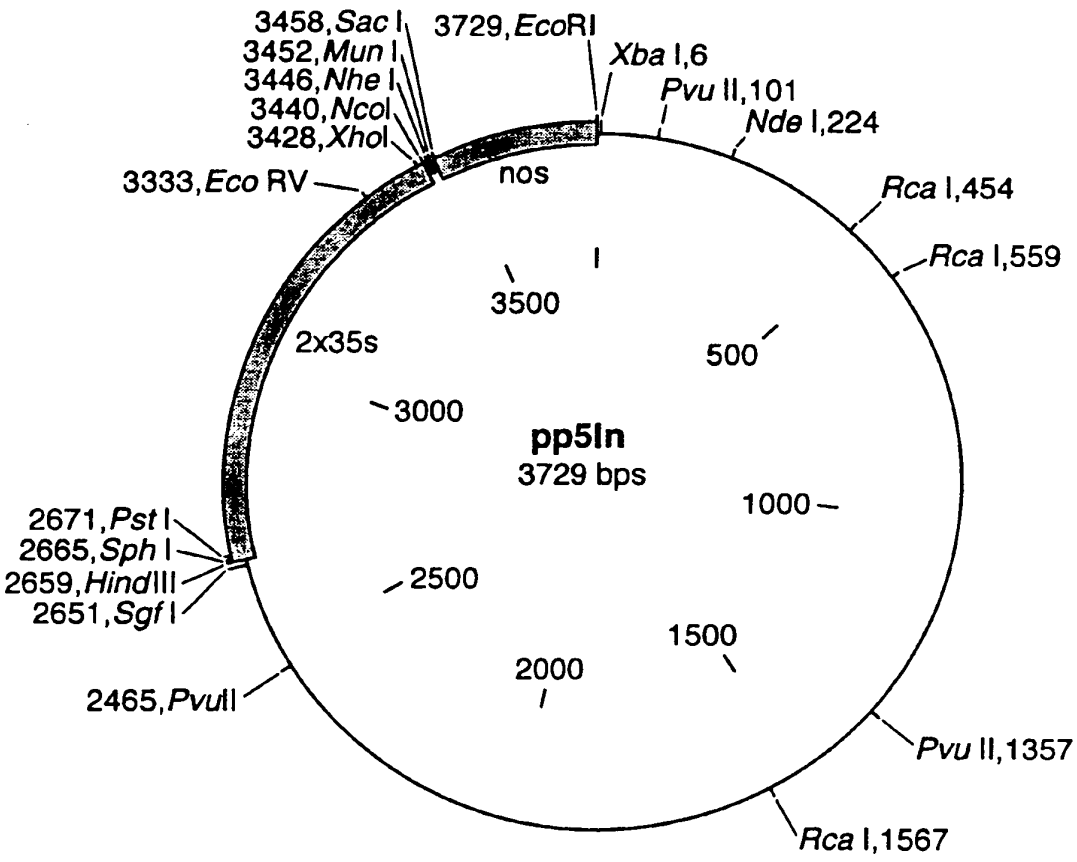


Fig.5.
pSJ34

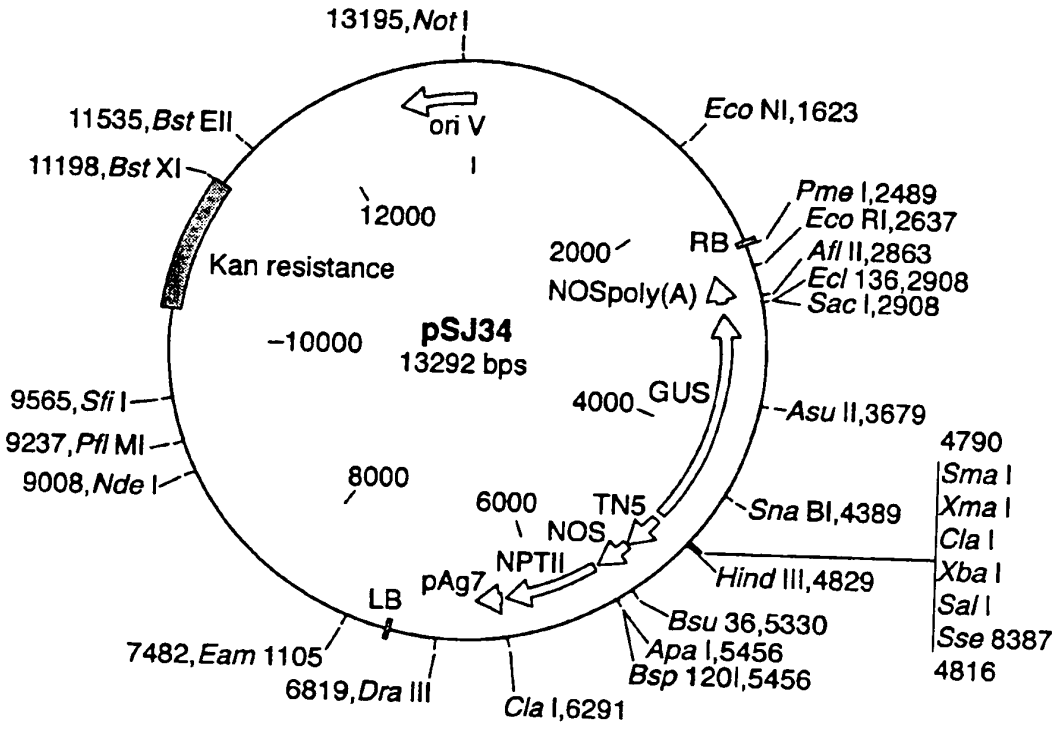

13195,Not I
ori V
I
Eco NI,1623
11535,Bst EII
11198,Bst XI
12000
Kan resistance
2000
RB
Pme I,2489
Eco RI,2637
Afl II,2863
Ecl 136,2908
Sac I,2908
NOSpoly(A)
-10000
pSJ34
13292 bps
4000
GUS
Asu II,3679
9565,Sfi I
9237,Pfl MI
9008,Nde I
8000
6000
TN5
NOS
NPTII
pAg7
LB
Sna BI,4389
4790
Sma I
Xma I
Cla I
Xba I
Sal I
Sse 8387
4816
Hind III,4829
Bsu 36,5330
Apa I,5456
Bsp 120I,5456
7482,Eam 1105
6819,Dra III
Cla I,6291

Fig.6.

Schematic drawing showing the construction of vector pNH5

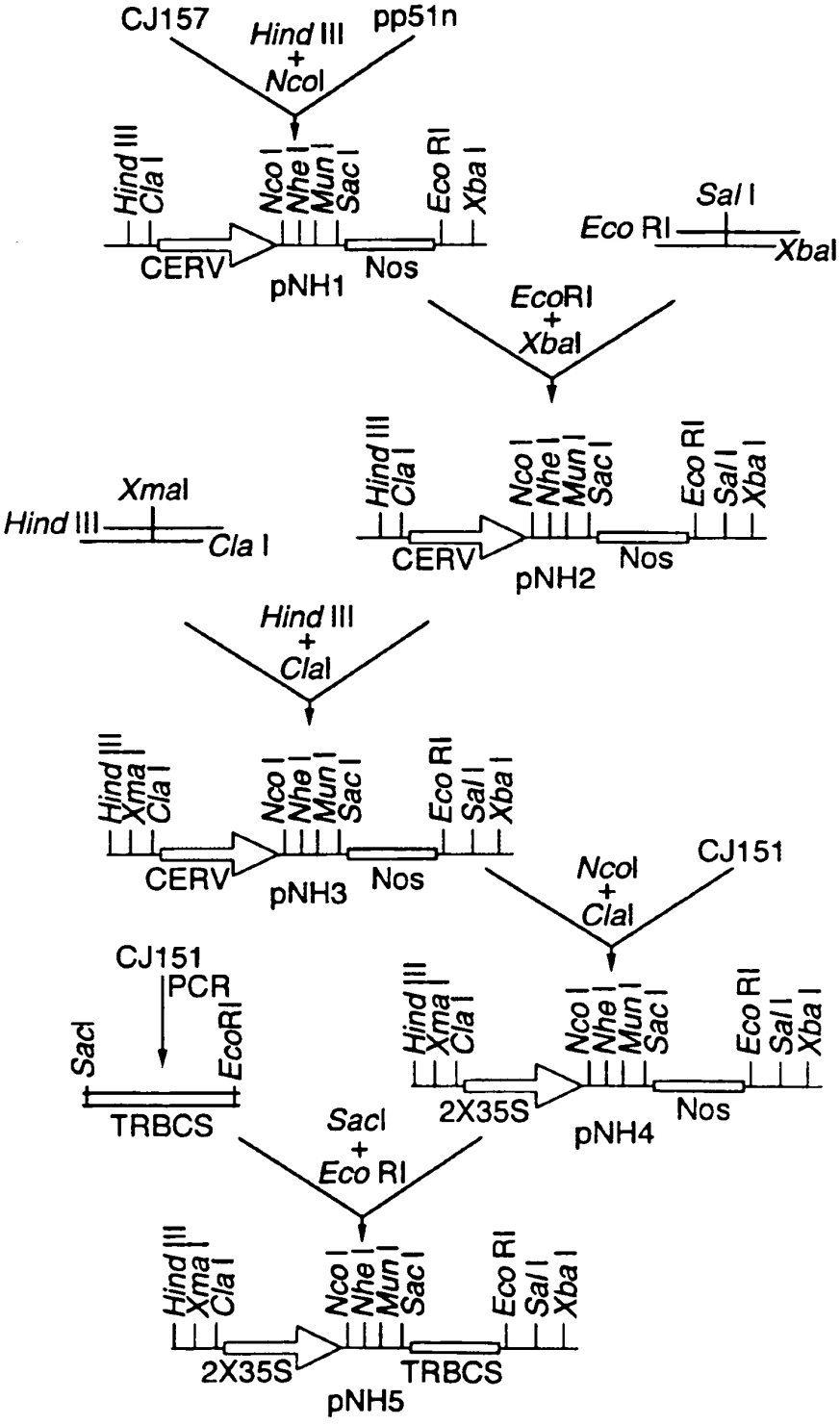


Fig.7.
Vector pNH16

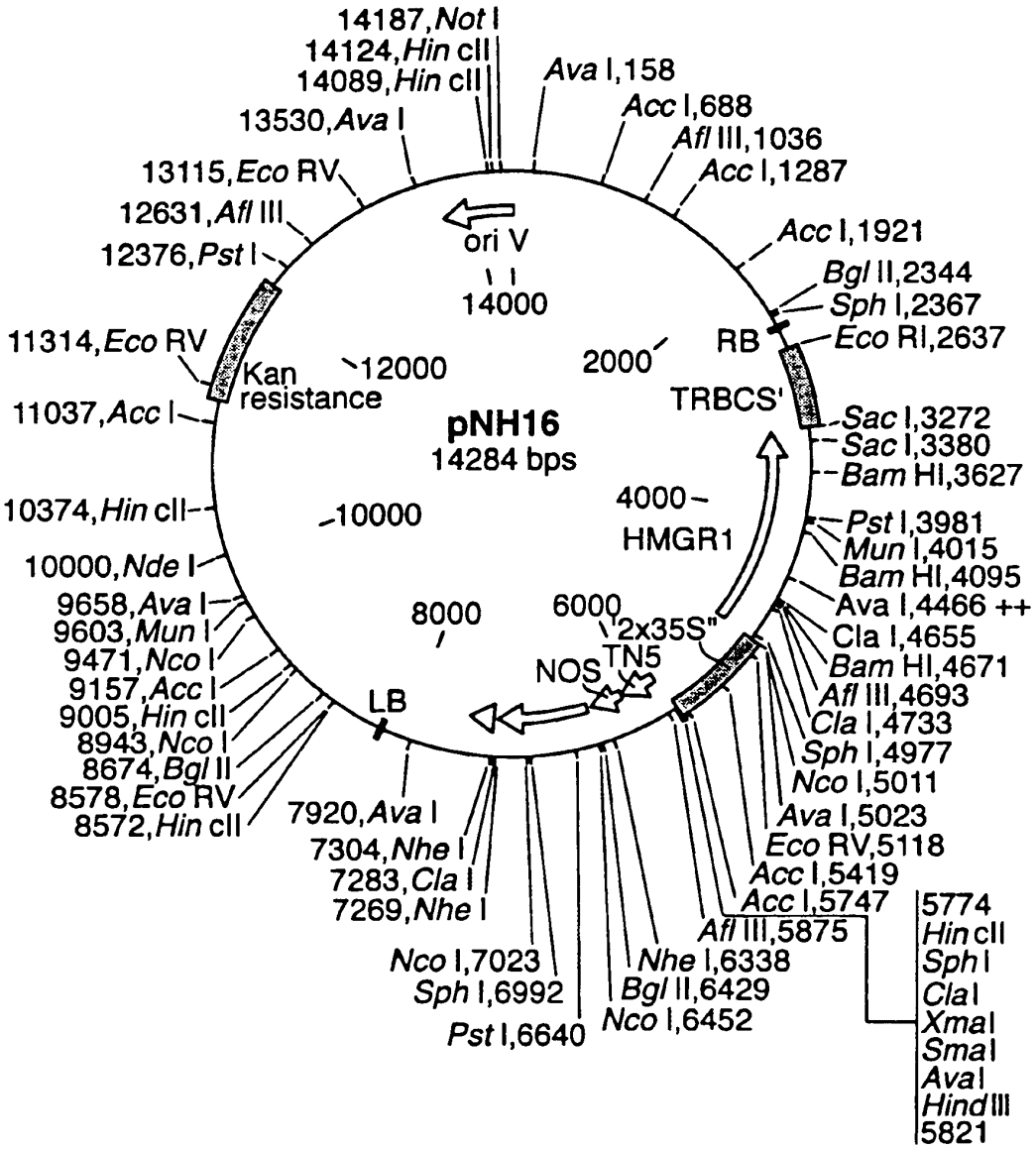


Fig.8.

Schematic representation of the construction of binary vector pNH16

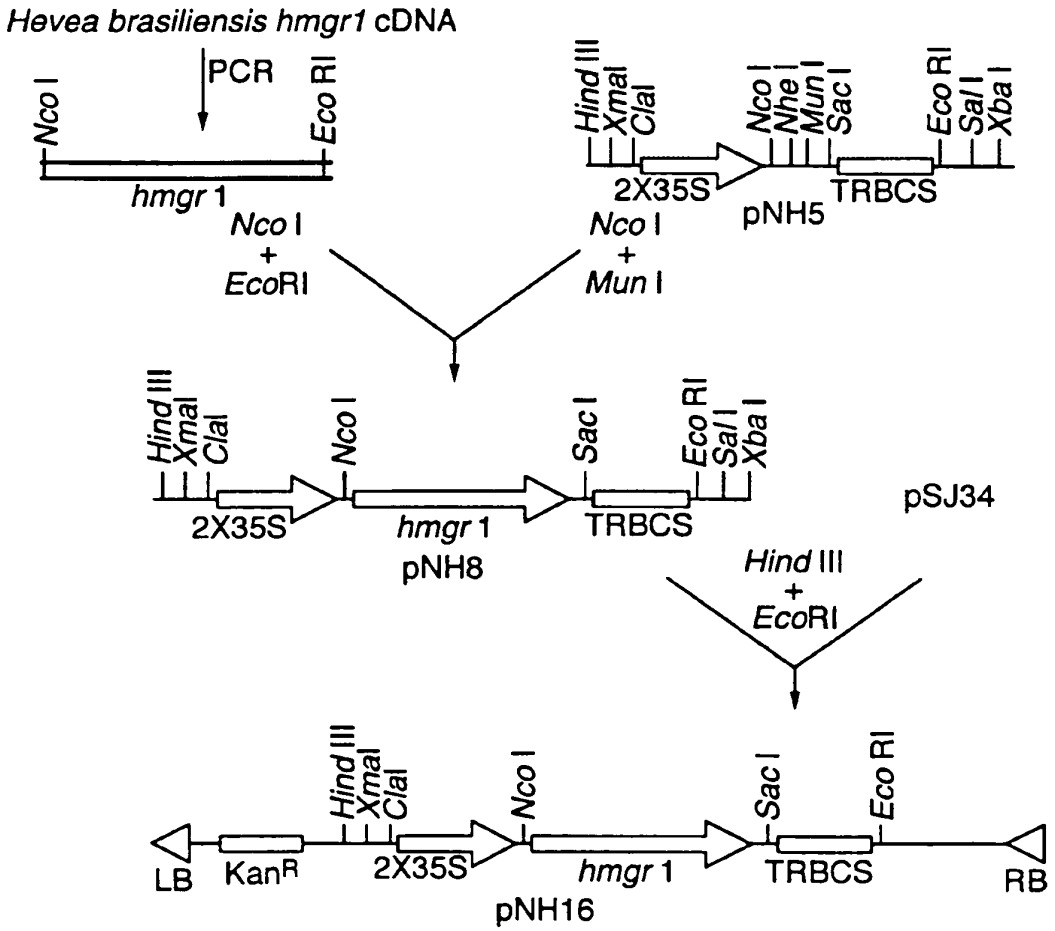


Fig.9.

Localisation of the sequencing PCR primers in
A pNH5, B pNH8 and C pNH16

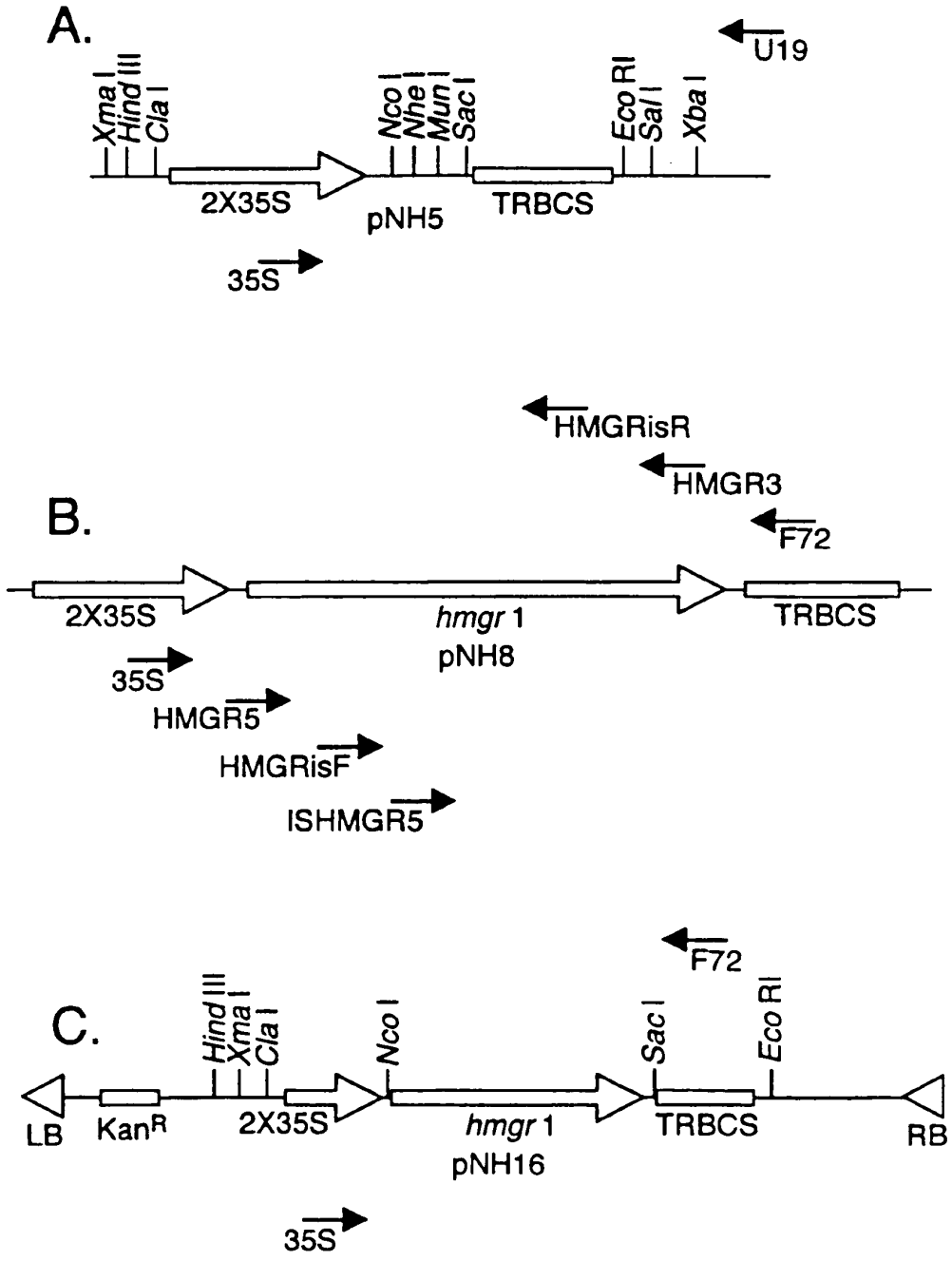


Fig.10.

Comparison between the published hmgr1 gene and the hmgr1 gene in pNH16

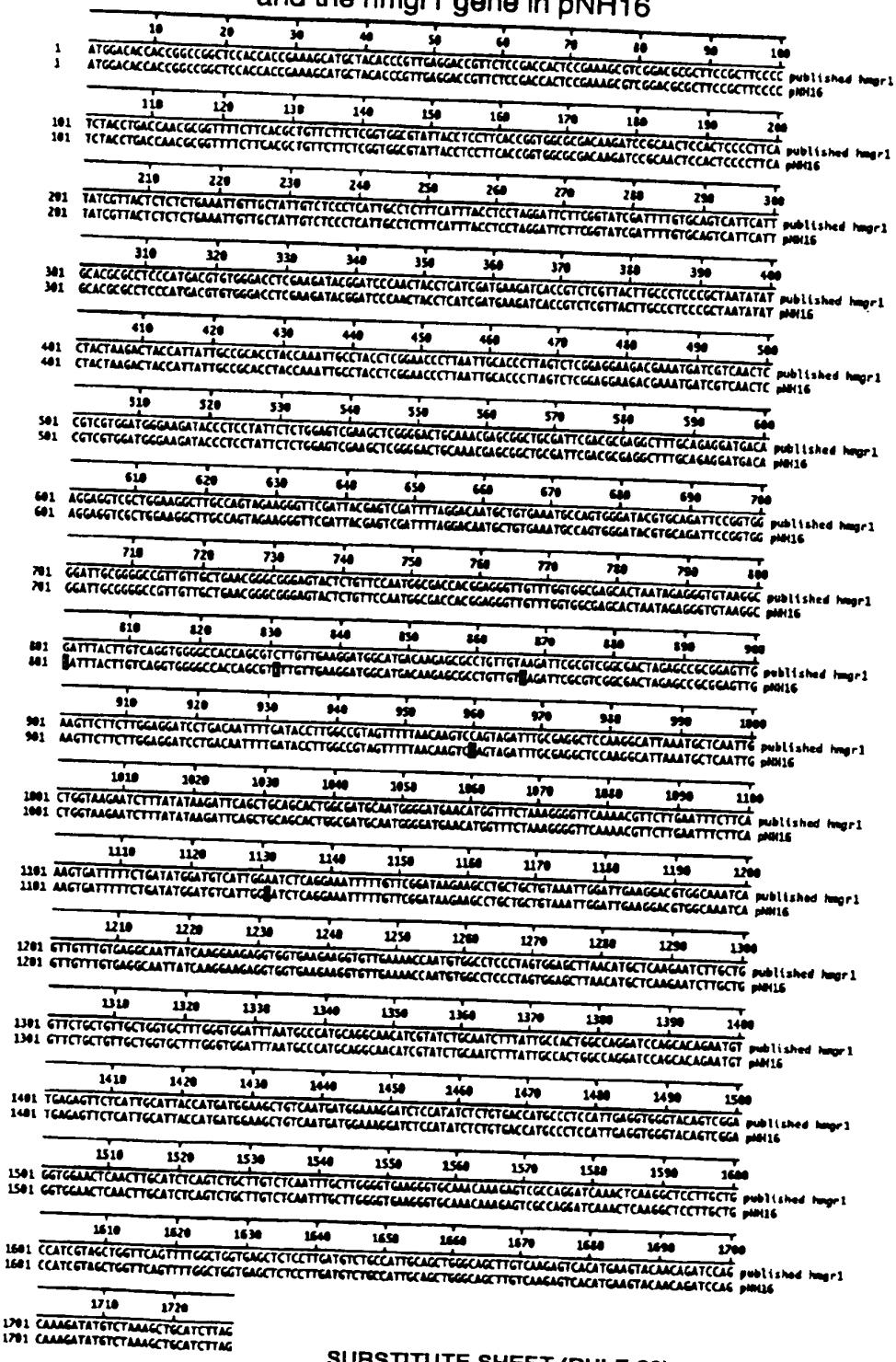


Fig.11A.

Hevea brasiliensis truncated HMGR sequence

```
   1-ATGGTTGCAC CCTTAGTCTC GGAGGAAGAC GAAATGATCG TCAACTCCGT CGTGGATGGG-60
     M  V  A  P  L  V  S  E  E  D  E  M  I  V  N  S  V  V  D  G

  61-AAGATACCCT CCTATTCTCT GGAGTCGAAG CTCGGGGACT GCAAACGAGC GGCTGCGATT-120
     K  I  P  S  Y  S  L  E  S  K  L  G  D  C  K  R  A  A  A  I

 121-CGACGCGAGG CTTTGCAGAG GATGACAAGG AGGTCGCTGG AAGGCTTGCC AGTAGAAGGG-180
     R  R  E  A  L  Q  R  M  T  R  R  S  L  E  G  L  P  V  E  G

 181-TTCGATTACG AGTCGATTTT AGGACAATGC TGTGAAATGC CAGTGGGATA CGTGCAGATT-240
     F  D  Y  E  S  I  L  G  Q  C  C  E  M  P  V  G  Y  V  Q  I

 241-CCGGTGGGGA TTGCGGGGCC GTTGTTGCTG AACGGGCGGG AGTACTCTGT TCCAATGGCG-300
     P  V  G  I  A  G  P  L  L  L  N  G  R  E  Y  S  V  P  M  A

 301-ACCACGGAGG GTTGTTTGGT GGCGAGCACT AATAGAGGGT GTAAGGCGAT TTACTTGTCA-360
     T  T  E  G  C  L  V  A  S  T  N  R  G  C  K  A  I  Y  L  S

 361-GGTGGGGCCA CCAGCGTCTT GTTGAAGGAT GGCATGACAA GAGCGCCTGT TGTAAGATTC-420
     G  G  A  T  S  V  L  L  K  D  G  M  T  R  A  P  V  V  R  F

 421-GCGTCGGCGA CTAGAGCCGC GGAGTTGAAG TTCTTCTTGG AGGATCCTGA CAATTTTGAT-480
     A  S  A  T  R  A  A  E  L  K  F  F  L  E  D  P  D  N  F  D

 481-ACCTTGGCCG TAGTTTTTAA CAAGTCCAGT AGATTTGCGA GGCTCCAAGG CATTAAATGC-540
     T  L  A  V  V  F  N  K  S  S  R  F  A  R  L  Q  G  I  K  C

 541-TCAATTGCTG GTAAGAATCT TTATATAAGA TTCAGCTGCA GCACTGGCGA TGCAATGGGG-600
     S  I  A  G  K  N  L  Y  I  R  F  S  C  S  T  G  D  A  M  G

 601-ATGAACATGG TTTCTAAAGG GGTTCAAAAC GTTCTTGAAT TTCTTCAAAG TGATTTTTCT-660
     M  N  M  V  S  K  G  V  Q  N  V  L  E  F  L  Q  S  D  F  S

 661-GATATGGATG TCATTGGAAT CTCAGGAAAT TTTTGTTCGG ATAAGAAGCC TGCTGCTGTA-720
     D  M  D  V  I  G  I  S  G  N  F  C  S  D  K  K  P  A  A  V

 721-AATTGGATTG AAGGACGTGG CAAATCAGTT GTTTGTGAGG CAATTATCAA GGAAGAGGTG-780
     N  W  I  E  G  R  G  K  S  V  V  C  E  A  I  I  K  E  E  V

 781-GTGAAGAAGG TGTTGAAAAC CAATGTGGCC TCCCTAGTGG AGCTTAACAT GCTCAAGAAT-840
     V  K  K  V  L  K  T  N  V  A  S  L  V  E  L  N  M  L  K  N

 841-CTTGCTGGTT CTGCTGTTGC TGGTGCTTTG GGTGGATTTA ATGCCCATGC AGGCAACATC-900
     L  A  G  S  A  V  A  G  A  L  G  G  F  N  A  H  A  G  N  I

 901-GTATCTGCAA TCTTTATTGC CACTGGCCAG GATCCAGCAC AGAATGTTGA GAGTTCTCAT-960
     V  S  A  I  F  I  A  T  G  Q  D  P  A  Q  N  V  E  S  S  H

 961-TGCATTACCA TGATGGAAGC TGTCAATGAT GGAAAGGATC TCCATATCTC TGTGACCATG-1020
     C  I  T  M  M  E  A  V  N  D  G  K  D  L  H  I  S  V  T  M

1021-CCCTCCATTG AGGTGGGTAC AGTCGGAGGT GGAACTCAAC TTGCATCTCA GTCTGCTTGT-1080
     P  S  I  E  V  G  T  V  G  G  G  T  Q  L  A  S  Q  S  A  C

1081-CTCAATTTGC TTGGGGTGAA GGGTGCAAAC AAAGAGTCGC CAGGATCAAA CTCAAGGCTC-1140
     L  N  L  L  G  V  K  G  A  N  K  E  S  P  G  S  N  S  R  L

1141-CTTGCTGCCA TCGTAGCTGG TTCAGTTTTGG CTGGTGAGC TCTCCTTGAT GTCTGCCATT-1200
```

Fig.11A.(Cont.)

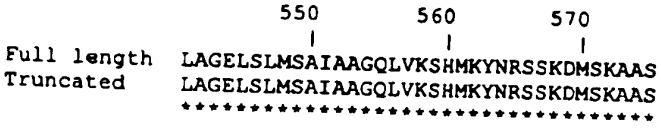
```
     L  A  A  I  V  A  G  S  V  L  A  G  E  L  S  L  M  S  A  I

1201-GCAGCTGGGC AGCTTGTCAA GAGTCACATG AAGTACAACA GATCCAGCAA AGATATGTCT-1260
     A  A  G  Q  L  V  K  S  H  M  K  Y  N  R  S  S  K  D  M  S

1261-AAAGCTGCAT CTTAG
     K  A  A  S  *
```

Fig.11b.

Alignment of H. Brasiliensis hmgr1 full length and truncated

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
Full length  MDTTGRLHHRKHATPVEDRSPTTPKASDALPLPLYLTNAVFFTLFFSVAYYLLHRWRDKI
Truncated    ------------------------------------------------------------

                    70        80        90       100       110       120
                     |         |         |         |         |         |
Full length  RNSTPLHIVTLSEIVAIVSLIASFIYLLGFFGIDFVQSFIARASHDVWDLEDTDPNYLID
Truncated    ------------------------------------------------------------

                   130       140       150       160       170       180
                     |         |         |         |         |         |
Full length  EDHRLVTCPPANISTKTTIIAAPTKLPTSEPLIAPLVSEEDEMIVNSVVDGKIPSYSLES
Truncated    ------------------------------MVAPLVSEEDEMIVNSVVDGKIPSYSLES
                                           ::**************************

                   190       200       210       220       230       240
                     |         |         |         |         |         |
Full length  KLGDCKRAAAIRREALQRMTRRSLEGLPVEGFDYESILGQCCEMPVGYVQIPVGIAGPLL
Truncated    KLGDCKRAAAIRREALQRMTRRSLEGLPVEGFDYESILGQCCEMPVGYVQIPVGIAGPLL
             ************************************************************

                   250       260       270       280       290       300
                     |         |         |         |         |         |
Full length  LNGREYSVPMATTEGCLVASTNRGCKAIYLSGGATSVLLKDGMTRAPVVRFASATRAAEL
Truncated    LNGREYSVPMATTEGCLVASTNRGCKAIYLSGGATSVLLKDGMTRAPVVRFASATRAAEL
             ************************************************************

                   310       320       330       340       350       360
                     |         |         |         |         |         |
Full length  KFFLEDPDNFDTLAVVFNKSSRFARLQGIKCSIAGKNLYIRFSCSTGDAMGMNMVSKGVQ
Truncated    KFFLEDPDNFDTLAVVFNKSSRFARLQGIKCSIAGKNLYIRFSCSTGDAMGMNMVSKGVQ
             ************************************************************

                   370       380       390       400       410       420
                     |         |         |         |         |         |
Full length  NVLEFLQSDFSDMDVIGISGNFCSDKKPAAVNWIEGRGKSVVCEAIIKEEVVKKVLKTNV
Truncated    NVLEFLQSDFSDMDVIGISGNFCSDKKPAAVNWIEGRGKSVVCEAIIKEEVVKKVLKTNV
             ************************************************************

                   430       440       450       460       470       480
                     |         |         |         |         |         |
Full length  ASLVELNMLKNLAGSAVAGALGGFNAHAGNIVSAIFIATGQDPAQNVESSHCITMMEAVN
Truncated    ASLVELNMLKNLAGSAVAGALGGFNAHAGNIVSAIFIATGQDPAQNVESSHCITMMEAVN
             ************************************************************

                   490       500       510       520       530       540
                     |         |         |         |         |         |
Full length  DGKDLHISVTMPSIEVGTVGGGTQLASQSACLNLLGVKGANKESPGSNSRLLAAIVAGSV
Truncated    DGKDLHISVTMPSIEVGTVGGGTQLASQSACLNLLGVKGANKESPGSNSRLLAAIVAGSV
             ************************************************************
```

Fig.11b.(Cont.)


550
560
570
Full length LAGELSLMSAIAAGQLVKSHMKYNRSSKDMSKAAS
Truncated LAGELSLMSAIAAGQLVKSHMKYNRSSKDMSKAAS

Saccharomyces cerevisiae truncated HMGR sequence

```
   1-ATGGGTCCTT TAGAAGAATT AGAAGCATTA TTAAGTAGTG GAAATACAAA ACAATTGAAG-60
     M  G  P  L  E  E  L  E  A  L  L  S  S  G  N  T  K  Q  L  K

  61-AACAAAGAGG TCGCTGCCTT GGTTATTCAC GGTAAGTTAC CTTTGTACGC TTTGGAGAAA-120
     N  K  E  V  A  A  L  V  I  H  G  K  L  P  L  Y  A  L  E  K

 121-AAATTAGGTG ATACTACGAG AGCGGTTGCG GTACGTAGGA AGGCTCTTTC AATTTTGGCA-180
     K  L  G  D  T  T  R  A  V  A  V  R  R  K  A  L  S  I  L  A

 181-GAAGCTCCTG TATTAGCATC TGATCGTTTA CCATATAAAA ATTATGACTA CGACCGCGTA-240
     E  A  P  V  L  A  S  D  R  L  P  Y  K  N  Y  D  Y  D  R  V

 241-TTTGGCGCTT GTTGTGAAAA TGTTATAGGT TACATGCCTT TGCCCGTTGG TGTTATAGGC-300
     F  G  A  C  C  E  N  V  I  G  Y  M  P  L  P  V  G  V  I  G

 301-CCCTTGGTTA TCGATGGTAC ATCTTATCAT ATACCAATGG CAACTACAGA GGGTTGTTTG-360
     P  L  V  I  D  G  T  S  Y  H  I  P  M  A  T  T  E  G  C  L

 361-GTAGCTTCTG CCATGCGTGG CTGTAAGGCA ATCAATGCTG GCGGTGGTGC AACAACTGTT-420
     V  A  S  A  M  R  G  C  K  A  I  N  A  G  G  G  A  T  T  V

 421-TTAACTAAGG ATGGTATGAC AAGAGGCCCA GTAGTCCGTT TCCCAACTTT GAAAAGATCT-480
     L  T  K  D  G  M  T  R  G  P  V  V  R  F  P  T  L  K  R  S

 481-GGTGCCTGTA AGATATGGTT AGACTCAGAA GAGGGACAAA ACGCAATTAA AAAAGCTTTT-540
     G  A  C  K  I  W  L  D  S  E  E  G  Q  N  A  I  K  K  A  F

 541-AACTCTACAT CAAGATTTGC ACGTCTGCAA CATATTCAAA CTTGTCTAGC AGGAGATTTA-600
     N  S  T  S  R  F  A  R  L  Q  H  I  Q  T  C  L  A  G  D  L

 601-CTCTTCATGA GATTTAGAAC AACTACTGGT GACGCAATGG GTATGAATAT GATTTCTAAA-660
     L  F  M  R  F  R  T  T  T  G  D  A  M  G  M  N  M  I  S  K

 661-GGTGTCGAAT ACTCATTAAA GCAAATGGTA GAAGAGTATG GCTGGGAAGA TATGGAGGTT-720
     G  V  E  Y  S  L  K  Q  M  V  E  E  Y  G  W  E  D  M  E  V

 721-GTCTCCGTTT CTGGTAACTA CTGTACCGAC AAAAAACCAG CTGCCATCAA CTGGATCGAA-780
     V  S  V  S  G  N  Y  C  T  D  K  K  P  A  A  I  N  W  I  E

 781-GGTCGTGGTA AGAGTGTCGT CGCAGAAGCT ACTATTCCTG GTGATGTTGT CAGAAAAGTG-840
     G  R  G  K  S  V  V  A  E  A  T  I  P  G  D  V  V  R  K  V

 841-TTAAAAAGTG ATGTTTCCGC ATTGGTTGAG TTGAACATTG CTAAGAATTT GGTTGGATCT-900
     L  K  S  D  V  S  A  L  V  E  L  N  I  A  K  N  L  V  G  S

 901-GCAATGGCTG GGTCTGTTGG TGGATTTAAC GCACATGCAG CTAATTTAGT GACAGCTGTT-960
     A  M  A  G  S  V  G  G  F  N  A  H  A  A  N  L  V  T  A  V

 961-TTCTTGGCAT TAGGACAAGA TCCTGCACAA AATGTTGAAA GTTCCAACTG TATAACATTG-1020
     F  L  A  L  G  Q  D  P  A  Q  N  V  E  S  S  N  C  I  T  L

1021-ATGAAAGAAG TGGACGGTGA TTTGAGAATT TCCGTATCCA TGCCATCCAT CGAAGTAGGT-1080
     M  K  E  V  D  G  D  L  R  I  S  V  S  M  P  S  I  E  V  G

1081-ACCATCGGTG GTGGTACTGT TCTAGAACCA CAAGGTGCCA TGTTGGACTT ATTAGGTGTA-1140
     T  I  G  G  G  T  V  L  E  P  Q  G  A  M  L  D  L  L  G  V

1141-AGAGGCCCGC ATGCTACCGC TCCTGGTACC AACGCACGTC AATTAGCAAG AATAGTTGCC-1200
```

```
      R  G  P  H  A  T  A  P  G  T  N  A  R  Q  L  A  R  I  V  A

1201-TGTGCCGTCT TGGCAGGTGA ATTATCCTTA TGTGCTGCCC TAGCAGCCGG CCATTTGGTT-1260
      C  A  V  L  A  G  E  L  S  L  C  A  A  L  A  A  G  H  L  V

1261-CAAAGTCATA TGACCCACAA CAGGAAACCT GCTGAACCAA CAAAACCTAA CAATTTGGAC-1320
      Q  S  H  M  T  H  N  R  K  P  A  E  P  T  K  P  N  N  L  D

1321-GCCACTGATA TAAATCGTTT GAAAGATGGG TCCGTCACCT GCATTAAATC CTAA
      A  T  D  I  N  R  L  K  D  G  S  V  T  C  I  K  S  *
```

Figure 12A (substitute; 03 Jan.2001)

Fig.12b.

Alignment of S. cerevisae hmgr1 full length and truncated

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
Full length  MPPLFKGLKQMAKPIAYVSRFSAKRPIHIILFSLIISAFAYLSVIQYYFNGWQLDSNSVF
Truncated    ------------------------------------------------------------

                    70        80        90       100       110       120
                     |         |         |         |         |         |
Full length  ETAPNKDSNTLFQECSHYYRDSSLDGWVSITAHEASELPAPHHYYLLNLNFNSPNETDSI
Truncated    ------------------------------------------------------------

                   130       140       150       160       170       180
                     |         |         |         |         |         |
Full length  PELANTVFEKDNTKYILQEDLSVSKEISSTDGTKWRLRSDRKSLFDVKTLAYSLYDVFSE
Truncated    ------------------------------------------------------------

                   190       200       210       220       230       240
                     |         |         |         |         |         |
Full length  NVTQADPFDVLIMVTAYLMMFYTIFGLFNDMRKTGSNFWLSASTVVNSASSLFLALYVTQ
Truncated    ------------------------------------------------------------

                   250       260       270       280       290       300
                     |         |         |         |         |         |
Full length  CILGKEVSALTLFEGLPFIVVVVGFKHKIKIAQYALEKFERVGLSKRITTDEIVFESVSE
Truncated    ------------------------------------------------------------

                   310       320       330       340       350       360
                     |         |         |         |         |         |
Full length  EGGRLIQDHLLCIFAFIGCSMYAHQLKTLTNFCILSAFILIFELILTPTFYSAILALRLE
Truncated    ------------------------------------------------------------

                   370       380       390       400       410       420
                     |         |         |         |         |         |
Full length  MNVIHRSTIIKQTLEEDGVVPSTARIISKAEKKSVSSFLNLSVVVIIMKLSVILLFVFIN
Truncated    ------------------------------------------------------------

                   430       440       450       460       470       480
                     |         |         |         |         |         |
Full length  FYNFGANWVNDAFNSLYFDKERVSLPDFITSNASENFKEQAIVSVTPLLYYKPIKSYQRI
Truncated    ------------------------------------------------------------

                   490       500       510       520       530       540
                     |         |         |         |         |         |
Full length  EDMVLLLLRNVSVAIRDRFVSKLVLSALVCSAVINVYLLNAARIHTSYTADQLVKTEVTK
Truncated    ------------------------------------------------------------

                   550       560       570       580       590       600
                     |         |         |         |         |         |
Full length  KSFTAPVQKASTPVLTNKTVISGSKVKSLSSAQSSSSGPSSSSEEDDSRDIESLDKKIRP
Truncated    ------------------------------------------------------------
Consensus                                                    ------------MGP
                                                                         *

                   610       620       630       640       650       660
                     |         |         |         |         |         |
Full length  LEELEALLSSGNTKQLKNKEVAALVIHGKLPLYALEKKLGDTTRAVAVRRKALSILAEAP
Truncated    LEELEALLSSGNTKQLKNKEVAALVIHGKLPLYALEKKLGDTTRAVAVRRKALSILAEAP
Consensus    ************************************************************
```

Fig.12b.(Cont.)

```
                    670        680        690        700        710        720
                     |          |          |          |          |          |
Full length  VLASDRLPYKNYDYDRVFGACCENVIGYMPLPVGVIGPLVIDGTSYHIPMATTEGCLVAS
Truncated    VLASDRLPYKNYDYDRVFGACCENVIGYMPLPVGVIGPLVIDGTSYHIPMATTEGCLVAS
Consensus    ************************************************************

                    730        740        750        760        770        780
                     |          |          |          |          |          |
Full length  AMRGCKAINAGGGATTVLTKDGMTRGPVVRFPTLKRSGACKIWLDSEEGQNAIKKAFNST
Truncated    AMRGCKAINAGGGATTVLTKDGMTRGPVVRFPTLKRSGACKIWLDSEEGQNAIKKAFNST
Consensus    ************************************************************

                    790        800        810        820        830        840
                     |          |          |          |          |          |
Full length  SRFARLQHIQTCLAGDLLFMRFRTTTGDAMGMNMISKGVEYSLKQMVEEYGWEDMEVVSV
Truncated    SRFARLQHIQTCLAGDLLFMRFRTTTGDAMGMNMISKGVEYSLKQMVEEYGWEDMEVVSV
Consensus    ************************************************************

                    850        860        870        880        890        900
                     |          |          |          |          |          |
Full length  SGNYCTDKKPAAINWIEGRGKSVVAEATIPGDVVRKVLKSDVSALVELNIAKNLVGSAMA
Truncated    SGNYCTDKKPAAINWIEGRGKSVVAEATIPGDVVRKVLKSDVSALVELNIAKNLVGSAMA
Consensus    ************************************************************

                    910        920        930        940        950        960
                     |          |          |          |          |          |
Full length  GSVGGFNAHAANLVTAVFLALGQDPAQNVESSNCITLMKEVDGDLRISVSMPSIEVGTIG
Truncated    GSVGGFNAHAANLVTAVFLALGQDPAQNVESSNCITLMKEVDGDLRISVSMPSIEVGTIG
Consensus    ************************************************************

                    970        980        990       1000       1010       1020
                     |          |          |          |          |          |
Full length  GGTVLEPQGAMLDLLGVRGPHATAPGTNARQLARIVACAVLAGELSLCAALAAGHLVQSH
Truncated    GGTVLEPQGAMLDLLGVRGPHATAPGTNARQLARIVACAVLAGELSLCAALAAGHLVQSH
Consensus    ************************************************************

                   1030       1040       1050
                     |          |          |
Full length  MTHNRKPAEPTKPNNLDATDINRLKDGSVTCIKS
Truncated    MTHNRKPAEPTKPNNLDATDINRLKDGSVTCIKS
Consensus    ***********************************
```

Fig.13.
Vector pMH3

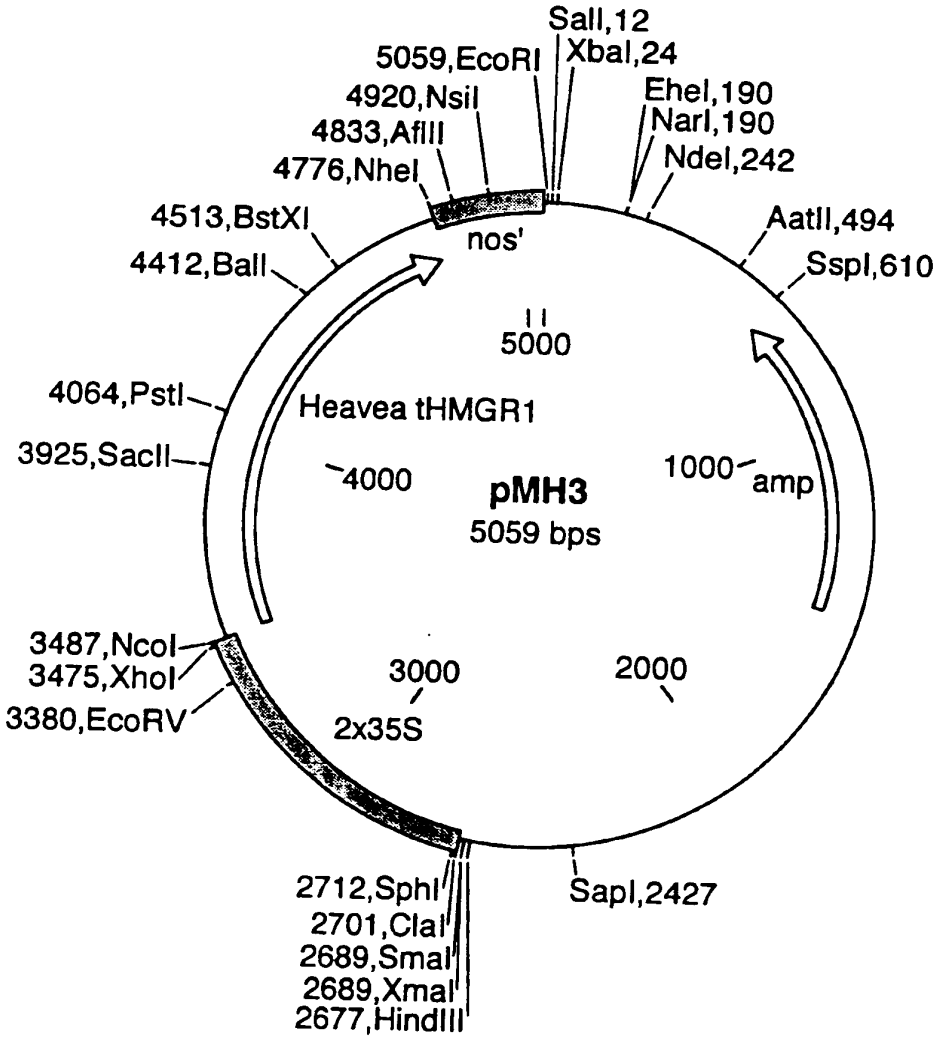


Fig.14.
Vector pMH4

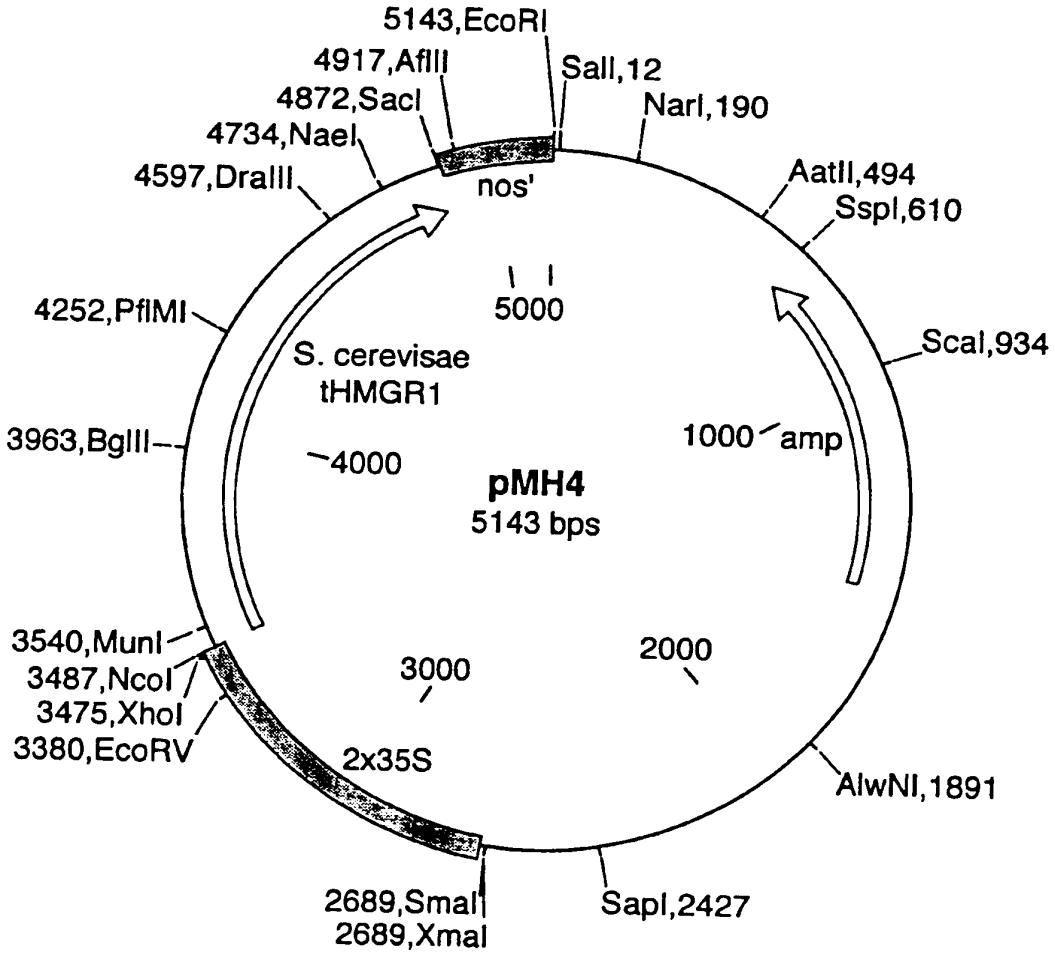


Fig.15.
Vector pMH5

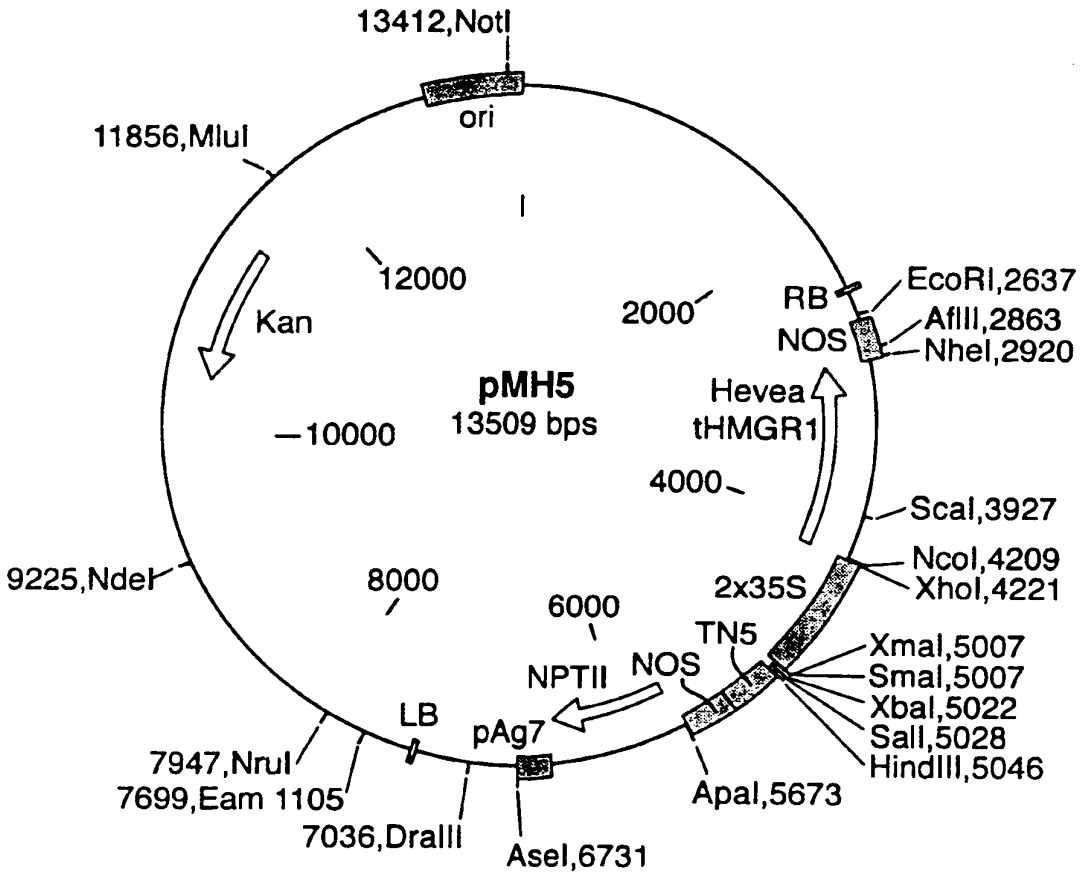


Fig.16.
Vector pMH6

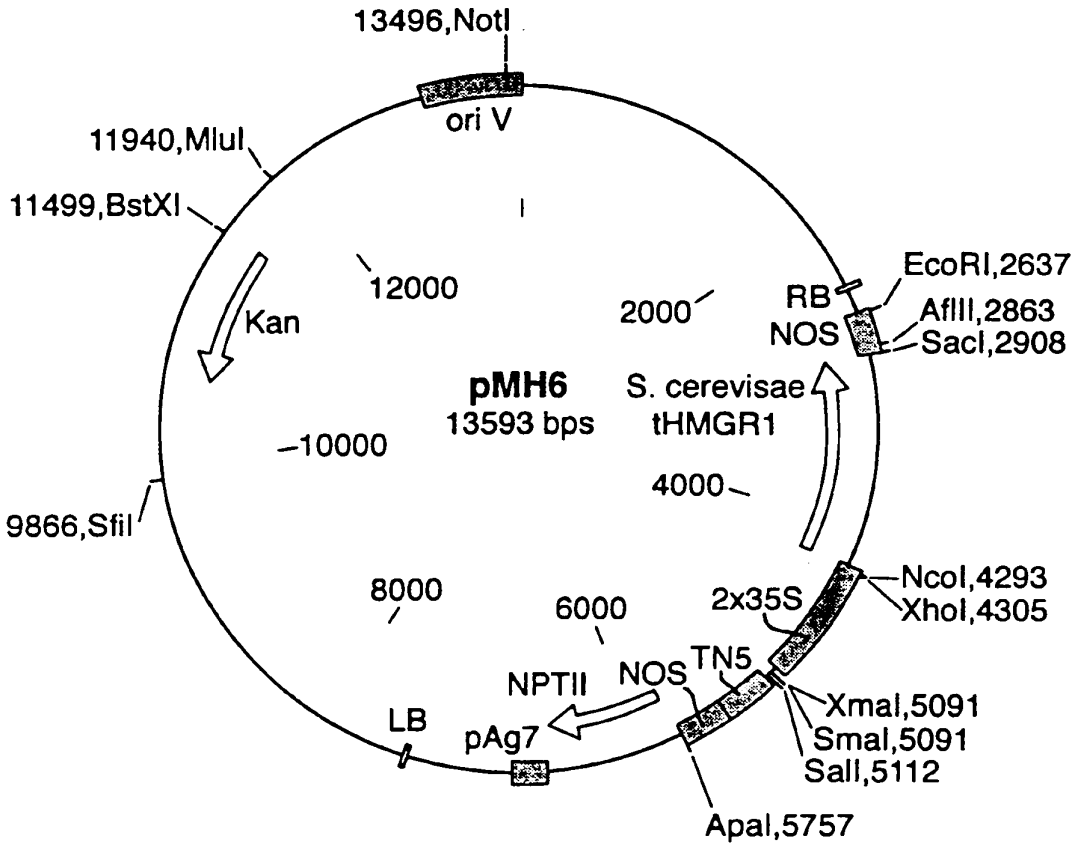
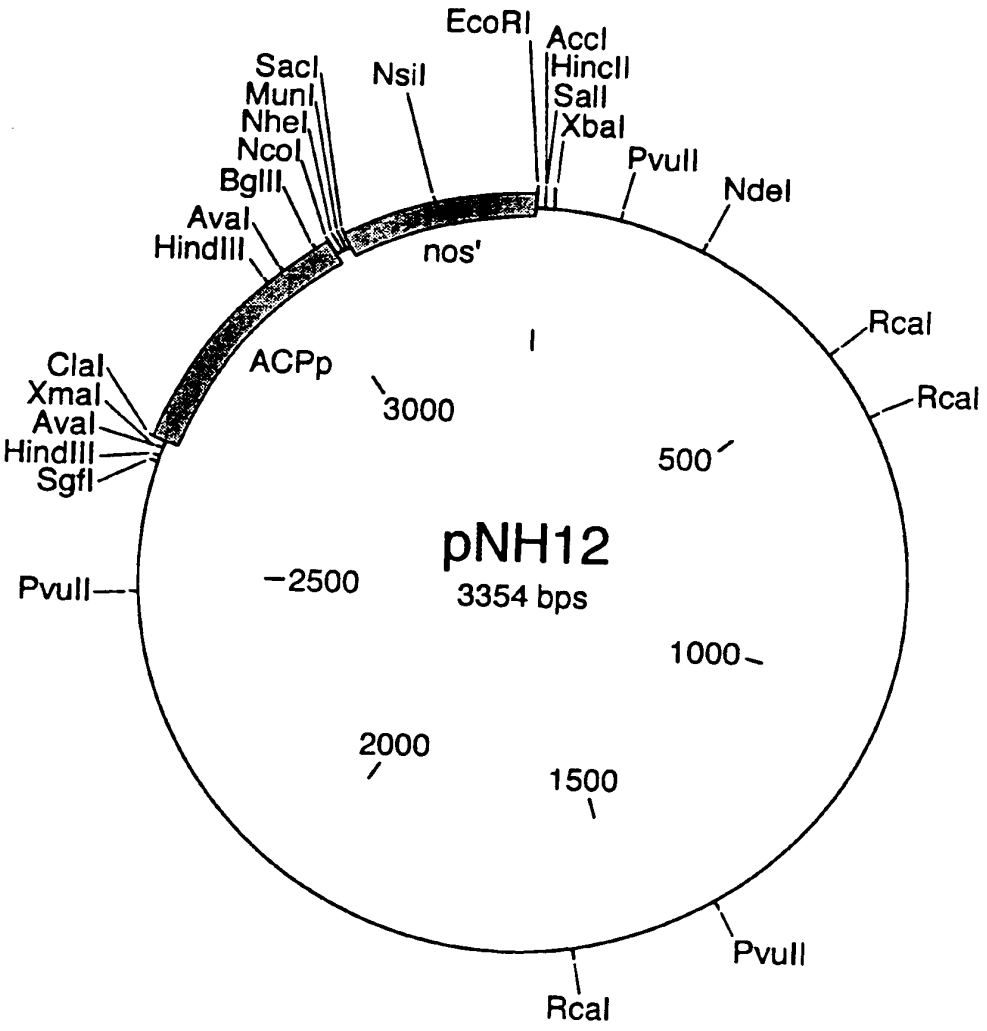


Fig.17.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SalI
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ClaI
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AvaI
HindIII
SgfI
AvaI
HindIII
SacI
MunI
NheI
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pNH12
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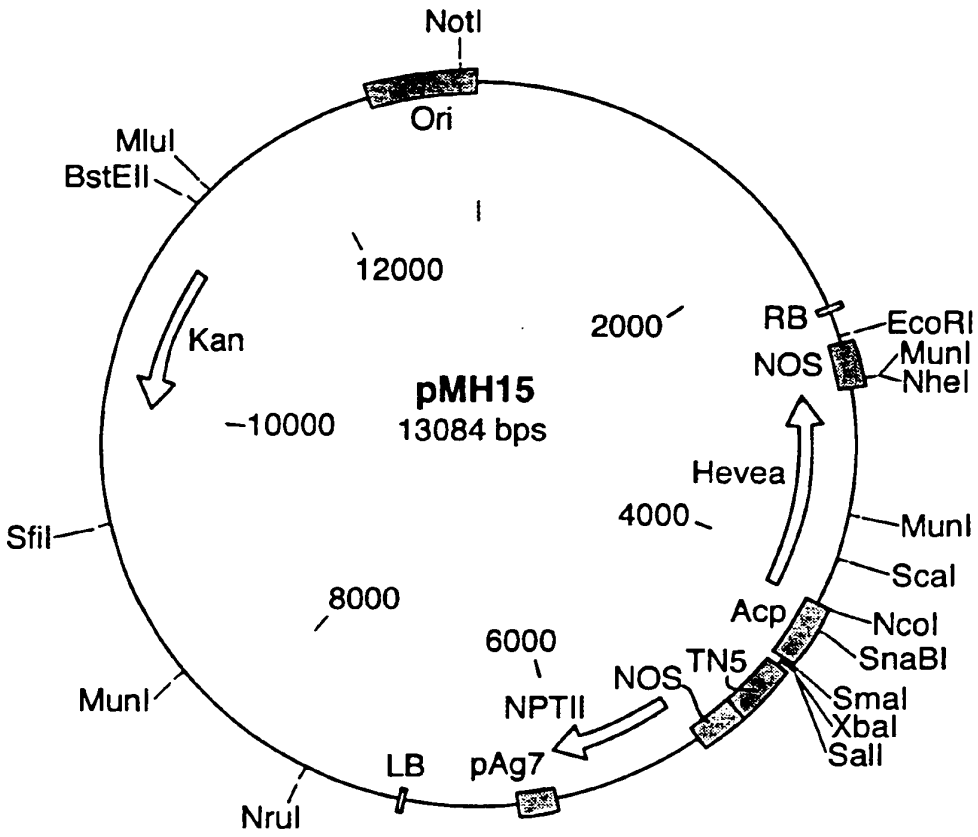
Fig.18.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MunI
SfiI
ScaI
8000
Acp
NcoI
6000
TN5
SnaBI
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SmaI
NPTII
XbaI
MunI
LB
pAg7
SalI
NruI

INTERNATIONAL SEARCH REPORT

Int. ...onal Application No
PCT/EP 00/09374

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2001	26/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Int[illegible]nal Application No
PCT/EP 00/09374

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